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Synergic Effect of Boswellic Acid and Ferulic Acid with Dextran Sulphate Sodium Induced Ulcerative Colitis in Zebra Fish

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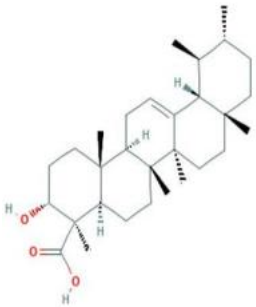
ABSTRACT:

To evaluate *in-silico, in-vitro* and *in-vivo* screening of boswelic acid and ferulic acid in Dextran sulfate sodium (DSS) induced ulcerative colitis in Zebra fish model.This Study involves the In silico study by the receptors will be selected for the studies in relation with the Ulcerative Colitis then it is docked by using Autodock 4.2.6 or Autodock Vienna. Then *In vitro study* is done by using the cell culture where the colorectal derived IEC lines HT-29 will be obtained. Cells were grown with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FCS in a humidified 5% CO2 atmosphere at 37°C. For signal-transduction experiments Rt-PCR should be performed. In vivo study is performed in the Zebra fish in a separate tank by injecting the Bosewellic acid. The study results suggest that B.serrata and F acid acts by inhibiting lipid peroxidation and its antioxidant effect is significant in the restoration of the enzymes. Thus, the results justify its use for the treatment of gastrointestinal diseases but more detailed studies should be conducted in order to evaluate the protective effect in humans.

Keywords: Boswellic acid, Dextran sulfate sodium, Ferulic acid, Ulcerative colitis

1. Introduction

Inflammatory bowel diseases (IBD) are intestinal diseases with uncertain aetiology. The dysregulation of mucosal immune acknowledge gut flora which inflame the bowel in the vulnerable hereditary individuals.[1] Chronic inflammation in IBD is designated in the gastro intestinal tract which involves genetic and environmental. It implicates the disease pathophysiology and included in classification of IBD. Ulcerative colitis (UC) affects the colon and rectum, with mucosal leukocyte penetration and superficial ulcers as symptoms..[4] Ulcerative colitis usually affects colon and intestinal superficial layers. The IBD onset can lead to complex and achieve interaction between all the factors. [5,6] The most common signs of IBD are stomach pain and bloody diarrhoea. Weight loss, fever, and anaemia are also possible symptoms. Symptoms are often slow to appear and can vary from mild to serious. Distinctive symptoms occur with time interval between flares. IBD complications encompass colon dilation abnormally (megacolon), eye, joints or liver and colon cancer inflammation.[7]



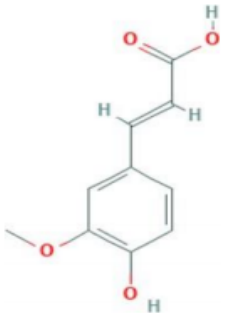


Figure1: Structure of Boswelic acid Figure 2: Structure of Ferulic acid

2. Materials and Methods

Chemical Collection

Boswellia serrata dry extract collected from TVS Biotech and the Ferulic acid extract collected from Avra synthesis

In-Vitro Cell Line Studies

The HT29 (Human colorectal adenocarcinoma) cell line was obtained from NCCS and cultured until confluent in medium supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), and streptomycin (100 g/ml) in a humidified atmosphere of 5% CO2. TPVG solution was used to dissociate the cell (0.2 percent trypsin, 0.02 percent EDTA, 0.05 percent glucose in PBS). The cells were checked for viability and centrifuged. A total of 50,000 cells were seeded per well in a 96-well plate and incubated for 24 hours at 37°C in a 5% CO2 incubator. Using respective media containing 10% FBS, the monolayer cell culture was trypsinized and the cell count was changed to 1.0 x 105 cells/ml. 100l of diluted cell suspension (50,000 cells/well) was applied to each well of the 96 well microtiter plate. The supernatant was flicked off after 24 hours, the monolayer was washed once with medium, and 100l of different concentrations of test drugs were applied to the partial monolayer in microtiter plates. The plate was then incubated for 24 hours at 37°C in a 5% CO2 atmosphere. The test solutions in the wells were discarded after incubation, and 100l MTT (5 mg/10 ml MTT in PBS) was applied to each well. The plate was incubated for 4 hours at 37 degrees Celsius in a 5% CO2 atmosphere. The supernatant was extracted, and 100 l of DMSO was applied to the plate, which was gently shaken to dissolve the formazan that had formed. A microplate reader was used to calculate the absorbance at a wavelength of 570 nm. The viability percentage was determined using the following formula: Sample abs/Control abs x 100 = percent of viability

Grouping of Animals

Table: 1 Animal Grouping and Treatment

Animal grouping	Treatment
Group I	Prednisolone (Std) + DSS
Group II	Vehicle
Group III	DSS+ saline
Group IV	Ferullic acid (20mg/kg) + DSS
Group V	Boswelic acid (80mg/kg) + DSS
Group VI	Boswelic acid + ferulic acid+ DSS

1: Grouping of animals

Experimental Design Induction of ulcerative colitis Dextran sodium sulphate (DSS) was used to induce Ulcerative colitis in zebrafish by adding DSS into water and was monitored daily. Body weight was also monitored in the zebra fish in all the groups. Treatment with chemical extract Boswellic acid: Dose: 80mg/kg Solubility: Dissolved in Ethanol 5mg/ml Dose was calculated for boswellic acid for 2 g body weight of the animal 22 Dose per animal was calculated 0.16mg Solubility was calculated 0.032 ml 50% of the dose = 0.24ml

Ferulic acid: Dose: 20mg/kg Solubility: Dissolved in Ethanol 10mg/ml Dose was calculated for ferullic acid for 2 g body weight of the animal Dose per animal was calculated 0.06 mg Solubility was calculated 0.06 ml 50% of the dose = 0.03

In-Vivo

• In a separate tank add ice cubes and place zebra fish into the tank to anaesthetize fish. Prepare a fresh solution of acetic acid. Find the anal opening by gently squeezing the proximal part of the abdomen. Inject 0.6 –1.0 L per 0.1 g of zebrafish gently. A 1 mL syringe attached to a small needle wrapped in silicon tubing with a diameter of less than 1 mm may be used for the injection.

3. Result

In-silico studies COX2 bound with 3-Ferulic Acid is a phytochemical antioxidant found naturally in the walls of the plant cells. It was docked with the human target CoX2. The binding energy score for this docking is -7.23 Kcal/mole and the inhibitory constant is 4.98 µM. The flexible residues that are involved in this docking are TYR 348, VAL 349, TYR 385, TRP 387, ARG 513, VAL 523. All these residues involved in the docking have close interaction with the ligand molecule after the docking also. The hydrogen bond is formed between the TRP 387 of the target protein and the ligand molecule. The desolv energy for the docking is -4.39 KCal/mole and the electrostatic energy is about -1.53 KCal/mole.

COX2-Boswellic Acid Boswellic Acid is the natural compound found in the resins of the plants in the genus of Boswellia. This boswellic acid is docked with the CoX2 target of the human. The binding energy score observed for this docking is -9.7 Kcal/mole. The inhibitory constant is 78 nM. The flexible residues that are involved in the docking are TYR 348, VAL 349, TYR 385, TRP 387, ARG 513, VAL 523. But only ARG 513 was involved in the formation of hydrogen bonds with the ligand molecule. The desolv energy and the electrostatic energy for this docking are -5.12 Kcal/mole and -0.82 Kcal/mole.

TNF - 3-ferulic acid 3-Ferulic Acid is a phytochemical antioxidant that was docked with TNF of humans. The binding energy score and the inhibitory constant for the docking are -5.22 Kcal/mole and 148.35 µM. The flexible residues involved in this docking are LEU 57, TYR

59, SER 60, GLN 61, LEU 120, TYR 119, and TYR 151. LEU 57, TYR 59, GLN 61, TYR 151 are closely involved in the interaction with ligand molecule after the docking. The hydrogen bond is formed in between ligand and SER 60, GLN 61, TYR 119 residues of the target protein. The desolv energy and the electrostatic energy of the docking are -3.75 Kcal/mole and -0.37 Kcal/mole.

TNF-Boswellic acid The observed binding energy score for the docking between the human TNF receptor and boswellic acid is -11.36 Kcal/mole. The inhibitory constant for this docking is -4.68 nM. LEU 57, TYR 59, SER 60, GLN 61, LEU 120, TYR 119, and TYR 151 are the residues involved in the docking. All the flexible residues that are involved in the docking are shown close interaction with the ligand molecule after the docking. SER 60, TYR 119 are the residues of the target involved in the formation of the hydrogen bond with the ligand. The dissolve energy and electrostatic energy observed for this docking are -5.58 Kcal/mole and -0.77 Kcal/mole.

Docking scores:

Query	Liver Toxicity		Metabolism						Membrane Transporters			Others			
				Cyp Inhibitors for											
	DILI	Cyto-toxicity	HLM	1A2	3A4	2D6	2C9	2C19	BBB	P-gp Inhibitor	P-gp Substrate	hERG Blocker	MMP	AMES	MRTD (mg/day)
	Yes	No	⊘	No	No	No	No	No	⊘	No	No	No	No	No	246
	⊘	⊘	⊘	No	No	No	No	No	⊘	Yes	No	⊘	No	⊘	268

Figure 3: ADMET Properties

Target Protein	PDB ID	Ligand	Binding Energy (kcal/mole)	Inhibitory constant	H-Bonding	Flexible residues
CoX2	5KIR	3 - Ferulic Acid	-7.23	4.98 uM	TRP 387	TYR 348 VAL 349 TYR 385 TRP 387 ARG 513 VAL 523
CoX2	5KIR	Boswellic Acid	-9.7	78 nM	ARG 513	TYR 348 VAL 349 TYR 385 TRP 387 ARG 513 VAL 523
TNF	6X81	3 - Ferulic Acid	-5.22	148.35 µM	SER 60 GLN 61 TYR 119	LEU 57 TYR 59 SER 60 GLN 61 LEU 120 TYR 119 TYR 151

TNF	6X81	Boswellic Acid	11.36	-4.68 nM	SER 60 TYR 119	LEU 57 TYR 59 SER 60 GLN 61 LEU 120 TYR 119 TYR 151

Table: 2 Docking Scores

ADMET PREDICTION OF THE DRUG MOLECULES USING

Table:3 ADMET Prediction

CONCENTRATION (µg/ml)	% of Viability	IC50 (µg/ml)
3.125	99.74	78.82
6.25	88.38	
12.5	86.05	
25	86.02	
50	55.65	
100	55.65	

The IC50 value of the given test sample B & F and the standard 5-Fluorouracil (5-FU) was found to be 78.82 µg and 2.94 µg, respectively

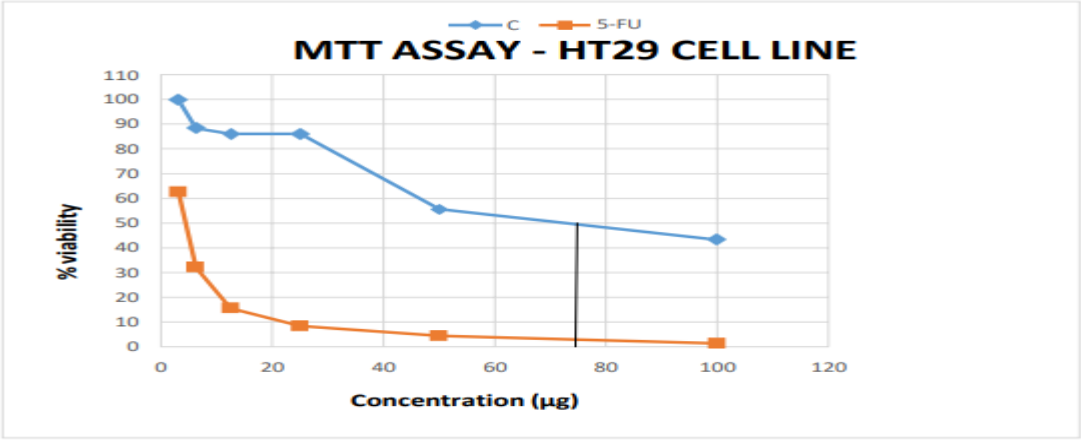
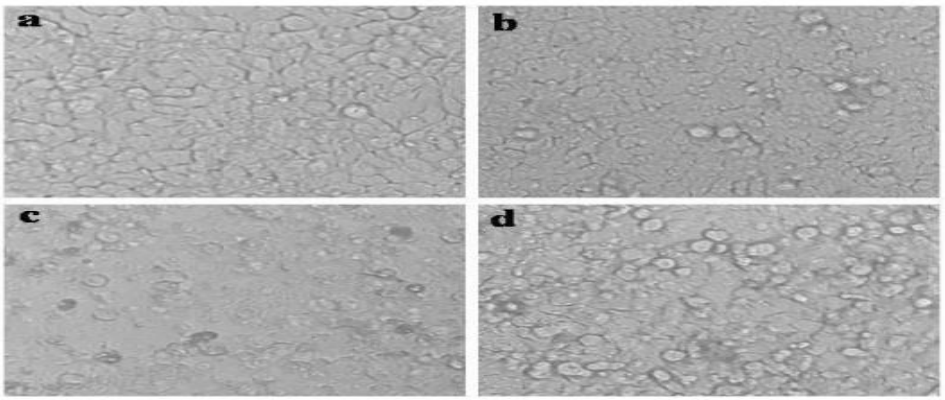


Figure 4: Percentage Cell Viability and IC50 Value

Table 4: MTT assay- - HT29 cell line MTT assay – HT29 cell line

ADME prediction	**Boswellic acid**	**Ferulic acid**
Forrmula	C10H10O4	C30H48O3
Molecular weight	194.18	456.70 g/mol
Molar Refractivity	51.63	136.91
Num. rotatable bonds	3	1
Num. H -bond acceptors	4	3
Num. H -bond donors	2	2
TPSA	66.76 Å²	57.53 Å²
Consensus Log Po/w	-1.36	6.09
Log S (ESOL)	-2.11	-7.81
Solubility	1.49e+00 mg/ml ; 7.68e -03 mol/l	7.08e-06 mg/ml 1.55e-08 mol/l
Class	Soluble	Low
GI absorption	High	No
BBB permeant	Yes	Yes
Log Kp (skin permeation)	-6.41 cm/s	-3.22 cm/s
Lipinski Drug Likeness	Yes	Yes;
Bioavailability Score	0.85	1 violation: MLOGP>4.15

Figure 5: HT29 Cell line



Histopathology:

Myeloperoxidase activity in tissue homogenate 10 volumes of ice-cold 50 mM sodium phosphate buffer, pH 6.0, containing 0.5 percent hexadecyl trimethyl ammonium bromide (Sigma aldrich) and 5 mM EDTA were used to homogenise wound tissue samples. The homogenate was sonicated on ice for 15 seconds, then freeze-thawed three times before centrifugation at 4°C for 30 minutes at 14 000 rpm. Lowry's standard approach was used to assess protein concentration. o-dianisidine oxidation was used to estimate MPO in the supernatant, and behaviour was determined based on the previous analysis. (A460 13.5)/amount of protein in sample = MPO activity (units/mg protein) (mg). A460: 3 minute change in absorbance at 460 nm. One unit of MPO activity was specified as the quantity capable of converting 1 mmol of H2O2 to water in 1 minute at 25 °C, using the coefficient 13.5 MPO Activity

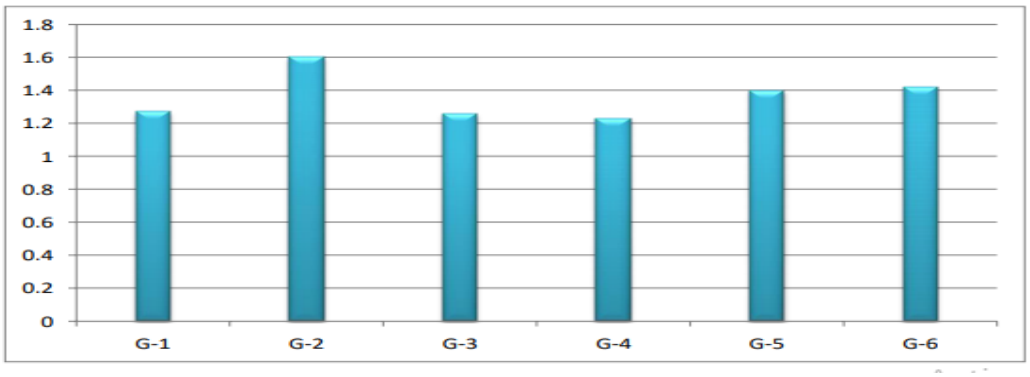


Figure 6: MPO Activity (units/mg protein)

TEST SAMPLE	Singlet	Duplicate	Triplicate	Mean		MPO activity	Protein content
G-1	0.373	0.375	0.376	0.374667	5.058	1.354	3.734
G-2	0.369	0.361	0.372	0.367333	4.959	1.591	3.116
G-3	0.317	0.316	0.323	0.318667	4.302	1.322	3.252
G-4	0.325	0.322	0.332	0.326333	4.405	1.262	3.489
G-5	0.391	0.399	0.396	0.395333	5.337	1.408	3.789
G-6	0.342	0.346	0.345	0.344333	4.648	1.453	3.198

Table 5: MPO Activity in singlet, duplicate and triplicate

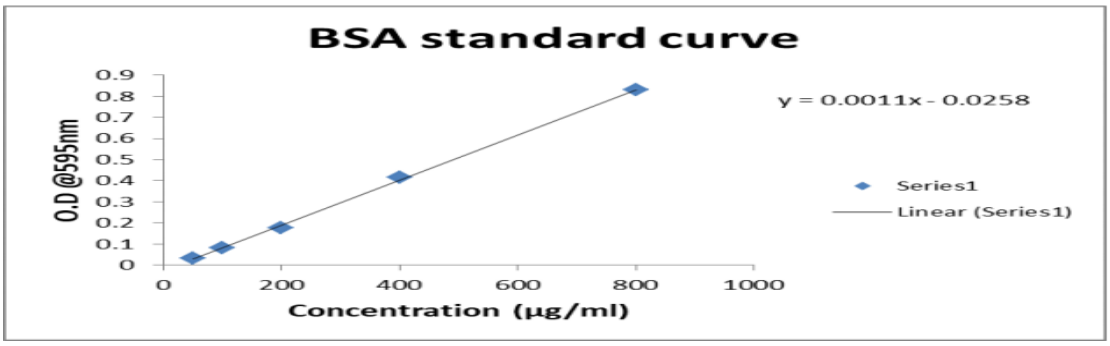
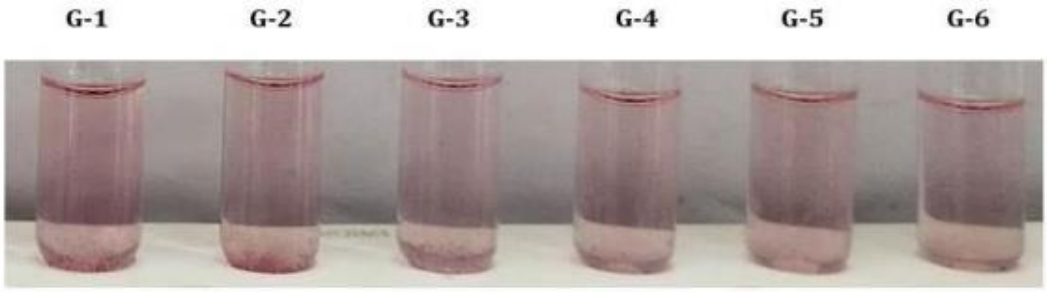


Figure 7: BSA Standard Curve

				Protein content
Groups	**OD (595)**		**10microL**	**100microL**
G-1	0.385	0.4108	373.4545455	3.734545455
G-2	0.317	0.3428	311.6363636	3.116363636
G-3	0.332	0.3578	325.2727273	3.252727273
G-4	0.358	0.3838	348.9090909	3.489090909
G-5	0.391	0.4168	378.9090909	3.789090909
G-6	0.326	0.3518	319.8181818	3.198181818

Table 6: Protein content of various concentrations

Figure 8: Groupings



Group 1 - Vehicle No histopathological changes are observed

Group 2 – Disease Control Severe loss of mucosa with disruption of intestinal villi, loss of goblet cells and leucocyte infiltration

Group 3 - Standard Minimal Loss of mucosa

Group 4 – F drug Mild Loss of Mucosa with disruption of intestinal villi, mild loss of goblet cells and leucocyte infiltration

Group 5 – B drug No histopathological changes are observed

Group 6 – F+B drug No histopathological changes are observed

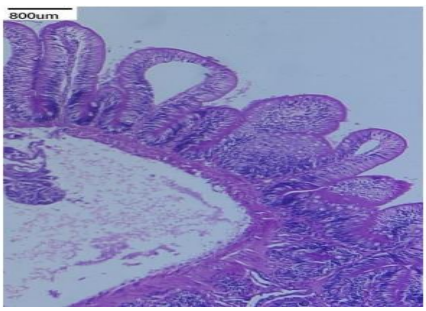


Figure 9: Vehicle Group

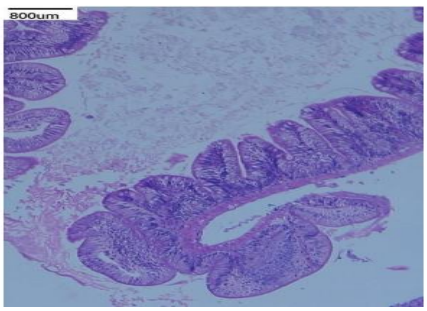


Figure 10: Disease Control

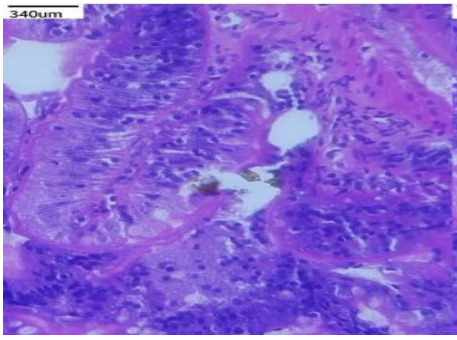


Figure 11: Standard

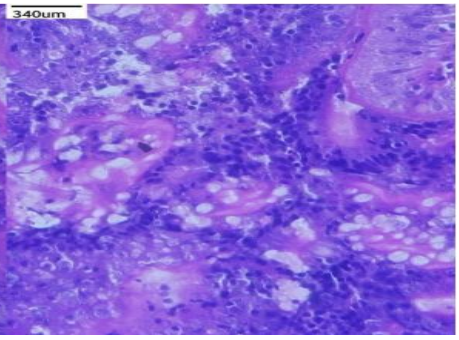


Figure 12: F-Drug

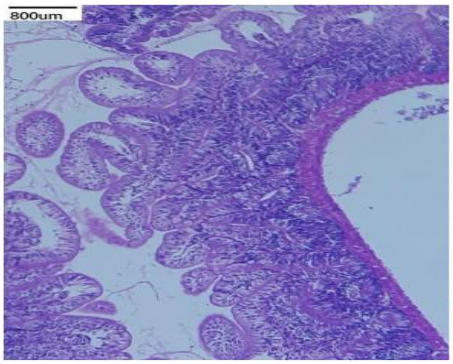


Figure 13: B-Drug

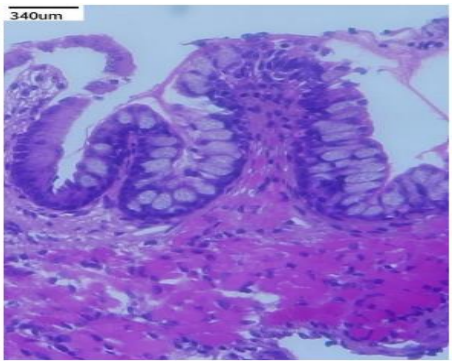


Figure 14: B + F Drug

4. Discussion

The induction of inflammatory bowel disease in rats using acetic acid is a well-known experimental model. Increased vasopermeability, prolonged oxidative stress, excessive neutrophil infiltration, and increased inflammatory mediator synthesis are all factors that contribute to the onset of IBD. [80-78] The disparity between offensive and defensive factors causes peptic ulcers, with the former including H. pylori and acid, and the latter including No, Growth factors, PG, and mucin. Despite its flaws, the Zebra fish model has proven to be superior to other versions in terms of intestinal inflammation. Because of the genetic and immunological similarities between humans and fish, mice can no longer be used as a model organism. Despite the fact that chemically induced models produce an inflammatory climate, they do not meet IBD requirements. Zebra fish are a low-cost species to experiment with and have a lot of potential for extrapolating effects. ROS (Reactive oxygen species) levels such as hydrogen peroxide, hydroxyl radical, hypochlorous acid, nitrogen species, and superoxide anion are well known in IBD patients, rendering oxidative stress a significant factor in the onset and maintenance of the disease.

Various parameters were all significantly increased in the colonic mucosa of animals induced by DSS (P 0.01) compared to standard controls. Nonetheless, these elevated parameters were all significantly reduced P 0.05/0.01 after model animals were treated with Boswelic acid and ferulic acid as specified in the experimental protocol, which was found to be very successful in treating colitis in zebra fish. Nitric oxide inhibits the relaxation of the internal anal sphincter, a smooth muscle. The release of iNOS in ulcerative colitis and subsequent increase of NO by stimulation of neurons and non-adrenergic non-cholinergic. In comparison to the other groups, the colitis-induced group has a substantial reduction in anal strain. [81] Dextran sodium

sulphate (DSS) causes an injury to the intestine, resulting in inflammation, which is the most common symptom of colitis. [82] Another important factor in the pathogenesis of IBD is abnormalities in arachidonic acid metabolism. COX-2 is activated in the metabolic pathway of arachidonic acid to produce excess PGE2 and TXB2 (important inflammatory mediators) in IBD, leading to edoema, bowel hypermia, and bowel dysfunction. TXB2 can also cause platelet aggregation, microthrombosis, vasoconstriction, and aggravation of inflammation. COX-2 or TXA2 synthesis inhibitors have
shown to be effective in the treatment of IBD. B. serrata contains antioxidants that have anti-inflammatory properties, such as preventing the synthesis of pro inflammatory cytokines like leukotrienes. They function by inhibiting the enzyme 5-lipoxygenase. Ferulic acid is an antioxidant-rich phytopolyphenol. In this analysis, ferulic acid was tested in vitro, in silico, and in vivo for potential anti-ulcer activity. Ferulic acid administration to pylorus ligated animals resulted in a decrease in ulcer index and volume of gastric contents, as well as a decrease in total and free acidity and lipid peroxidation. According to various studies, an imbalance in the mucosal immune system leads to the production of inflammatory mediators such as reactive oxygen species (ROS) and cytokines (TNF, IL-1B, and IL-6) that induce chronic inflammation, lesions over the colonic mucosa, ulceration, and leukocyte infiltration. When a haptenizing (inflammatory) agent like Dextran sodium sulphate (DSS) or TNBS is given, it causes delayed-type hypersensitivity and IL12-mediated Th1 T cell transmural colitis, allowing the effects of various immune-inflammatory inhibitory agents to be studied.
In the current research, we assessed various biochemicals, molecular, and histological changes in the zebra fish model of DSS-induced colitis to see whether FA and BA could help. We used the zebra fish model to see whether the compounds Boswelic acid and Ferulic acid reduced the effects of Dextran sodium sulphate on experimental colitis, as measured by myeloperoxidase function, MTT assay-HT29 cell line, percent of viability, and in-silico docking activities as key parameters in determining the magnitude of colon inflammation in IBD. Thus, the anti inflammatory ability of ferulic acid was investigated in zebra fish colonic damage caused by Dextran sodium sulphate (DSS) by analysing changes in biochemical, histological, and in-vivo parameters. DSS also significantly increased TNF-, IL-1, and IL-6 colonic mRNA expression, promoting an enhanced immune inflammatory response. As a result, FA has an immunomodulatory effect on intestinal inflammation. The antioxidants superoxide dismutase (an endogenous enzyme) and glutathione (a non enzymatic antioxidant) work together to protect cells from increased H2O2 output. The antioxidant system, which includes both enzymatic and non-enzymatic substances that act to prevent oxidative damage, is a compensatory mechanism for the oxidation method. To compensate for the damage caused by acetic acid action in the animal intestine, we found a substantial increase in SOD activity in the colitis population. SOD is a key enzyme in cellular redox balance, which protects tissues from oxidative damage. Animals with colitis treated 43 with B. serrata and ferulic acids, on the other hand, had SOD enzyme activity levels that were almost identical to the control group. A large number of studies have shown that IBD promotes oxidative stress and iNOS behaviour, resulting in a pathological cascade of free radical reactions and the development of further oxidative free radicals, such as peroxynitrite (ONOO-), which impairs cell structure and function. It is widely assumed that neutrophils (the core dogma for the development of superoxide anion and a cascade of various reactive species) trigger an oxidative injury cycle that culminates in tissue necrosis and mucosal dysfunction. Oxidative stress is a major mediator of the pathogenesis of ulcerative colitis, according to research conducted at both the preclinical and clinical levels.GSH (non-enzymatic antioxidant) has a high electrondonating potential and controls the redox state of the cell through a variety of mechanisms, including ROS scavenging and maintaining the reduced state of the enzyme GSH peroxidase. We believe that the

antioxidant activity of B. serrata and ferulic acid was successful in reducing the production of reactive oxygen species.

5. Conclusion

In conclusion, the study results suggest that B.serrata and F acid acts by inhibiting lipid peroxidation and its antioxidant effect is significant in the restoration of the enzymes. Thus, the results justify its use for the treatment of gastrointestinal diseases but more detailed studies should be conducted in order to evaluate the protective effect in humans. In summary, the results of this study show that intra-colonic treatment with BA and FA can ameliorate the pathological changes 46 of experimental colitis in zebra fish suggesting it to serve as an effective therapeutic agent for the treatment of IBD. Nonetheless, more systemic studies are necessary to extrapolate these results in higher mammals. Further, molecular docking of FA and BA with cyclooxygenase-2 was performed by AutoDock 4.2.3 program and we demonstrated that it binds with TNF-α COX-2 enzyme in the hydrophobic pocket stabilized mainly by two hydrogen bonds. This study has tremendous scope in the pharmaceutical industry and biology for the designing of novel inhibitors for TNF-α and COX-2 enzyme.

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