



ANTIOXIDANT AND ANTI-ULCERATIVE COLITIS EFFECT OF POLYHERBAL EXTRACT IN DSS INDUCED ZEBRAFISH MODEL

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Abstract:

Inflammatory bowel disease (IBD) is a chronic, recurrent, and remitting inflammatory disease with unclear etiology. As a clinically frequent disease, it can affect individuals throughout their lives, with multiple complications. Unfortunately, traditional murine models are not efficient for the further study of IBD. The zebrafish model has been used to study the composition of intestinal microbiota, novel genes, and therapeutic approaches. The pathogenesis of IBD is still unclear and many risk factors remain unidentified. Conventional treatment of ulcerative colitis is accompanied by a certain number of adverse effects. The use of herbal therapy in inflammatory bowel disease (IBD) is increasing worldwide. The present study was to evaluate the antioxidant activity using DPPH assay and anti-ulcerative colitis of polyherbal extract (*Curcuma longa*, *Andrographis paniculate*, *Cynodon dactylon* and *Citrus limon*) in DSS (Dextran sodium sulphate) induced Zebrafish model. 0.25% DSS given to the zebrafish for 7 days. On 8th day given for histopathological examination. The phytoconstituents present in the polyherbal extract was confirmed by FTIR and HPTLC. Presence of Phytoconstituents Flavonoids, Tannins, Triterpenoids exhibits a substantial reduction of internal inflammation and reduce intestinal mucosa damage. Herbal therapies exert their therapeutic benefit by different mechanisms including immune regulation, antioxidant activity.

Keywords: Zebrafish, Inflammatory bowel disease, Murine model, Polyherbal extract, Dextran sodium sulphate.

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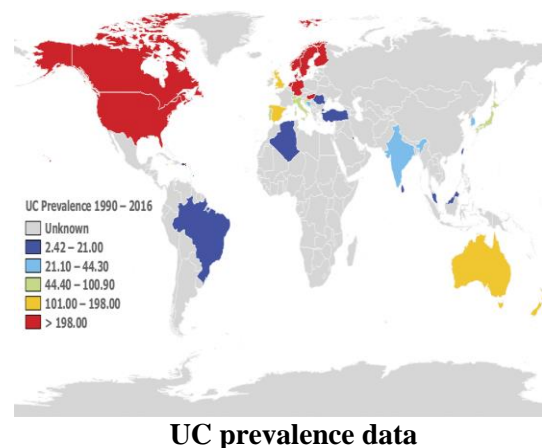
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I. INTRODUCTION:

- [1]. Inflammatory bowel disease is a chronic immunologically mediated disease of gastro intestinal tract distinct by means of events of reversion and decline.
- [2]. There are two major recognized types of the disease, Crohn's disease and ulcerative colitis. The two major subtypes share a hypothesized etiology of genetic proneness wherein different genes linked to each environmental factors or exposures and alterations of the gut micro -biomes that subsidies to the indulgent of the disease.
- [3]. IBD is characterized by intestinal inflammation and mucosal tissue damage initiated and perpetuated by a dysregulated immune response along with several intra and extra intestinal manifestations.
- [4]. These conditions are chronic, relapsing and remitting at regular intervals which results in diarrhoea and abdominal pain. Research studies concerning IBD suggests that the immune system is altered in people with these conditions. The other factors include genetic predisposition and environmental factors.
- [5]. Inflammatory bowel disease is much common in white people of European descent living in industrial regions of the western world. The resumed modifications in environmental factors, including contamination diets, way of living factors, and uses of medication, have contributed to alterations in the universality of the disease.
- [6]. In a survey, they came across the most convincing studies about the essential uses of natural products and plants in the treatment of IBD, that poses multiple cellular and molecular mechanism which in turn, explain their Anti-inflammatory and immune modulation action, side effect of novel drugs and their extensive uses where some of them are life- threatening.
- [7]. Ulcerative colitis causes ulcers and inflammation of the lining of the colon. It almost always involves the rectum and usually causes bloody diarrhoea.
- [8]. Crohn's disease is an inflammation that extends into the deeper layers of the intestinal wall. The disease is either limited to one or more segments of small intestine (usually the ileum) or involves both the ileum and the colon (ileocolitis). In some, crohn's disease is also confined to the colon. Sometimes inflammation may also affect the mouth, oesophagus, stomach, duodenum, appendix, or anus.
- [9]. Left sided inflammatory bowel disease point out illness including the colon proximal to the lineal flexure and include pan-colitis, wherever total colon is concerned. Ulcerative colitis or inflammatory bowel sickness could be a womb-tomb disease that's related to vital morbidity. Associate degree calculates five hundredth of individuals with colitis can have a minimum of one relapse per year.
- [10]. Approximately twenty-fifth of individuals with ulcerative inflammatory bowel disease possess single or additional event of intense severe colitis in their lifespan, along twenty-ninth colectomy rate. Though lethality scales have upgraded steadily go thirty years, intense extreme inflammatory bowel disease still features a range up to twenty.
- [11]. Lethality is altered by temporal order of interventions, as well as medical care and colectomy. The foremost recent UK audit incontestable associate degree overall GB national mortality of 0.8%. Presently, the frequency every year for CD is getting higher in northern part of America and European countries with 20.3 and 2403 per 100,000 per person. IBD seems to be more developing in France, Scotland, New Zealand, Australia and in Asia.

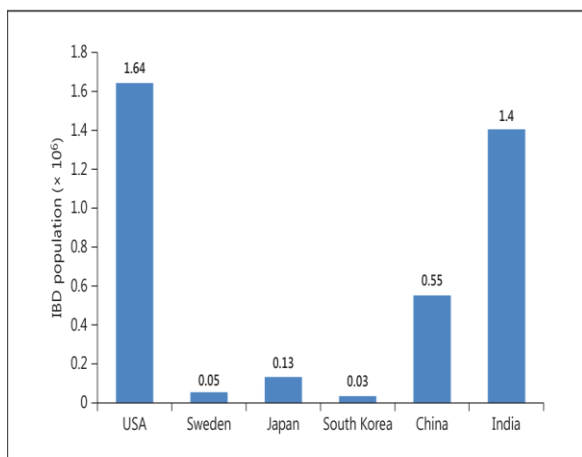


II. MATERIALS AND METHODS PROCUREMENT:

The four Plants were collected near the local area and identified. These Plants are authenticated by professor P. JAYARAMAN PhD, Director institute of Herbal Botany .Plant Anatomy Research Centre no 4 Sakthi Nagar west Tambaram Chennai-15,India in the month of February 2020 and Registered number of the PARC/2020/4216, PARC/2020/4217, PARC/2020 /4218, PARC/2020/4219. Disease burden of UC and CD in India based on two population studies was conducted by Khosla et

al. and Stood et al. in 1984 and 1999. It showed a prevalence of 42.8 and 44.3 per 100,000 people. A recent epidemiological study, entitled "The Asia-pacific Crohn's and Colitis Epidemiologic Study (ACCESS) who studied IBD and says that the incidence of IBD, UC and CD from Asia was 1.37, 0.76 and 0.54 per 100,000, whereas in Australia it was 23.67, 7.33 and 14.00 respectively.

[11,12] The overall estimated IBD population in India in 2010 came out to be 1.4 million, which was the second highest number after the USA (with 1.64 million). Therefore, although the disease prevalence in India is lower than in the west, with a population of more than 120 million, the total IBD population in India is among the largest across the globe. In past years, many herbal products have proven to be cost effective and they strengthen the beneficial effect of the drug. According to WHO estimates that 78.9% of the population of some Asian parts and parts of African countries presently use herbal drug for some of basic health care such as Curcumin, Aloe Vera, Boswellia serrata are used as therapeutic agents in treating IBD. The objective of the present study describes the extraction and preclinical evaluation of polyherbal extract (Curcuma longa, Andrographis paniculate, Cynodon dactylon and citrus limon) against DDS induced colitis in zebrafish model.



UC prevalence across globe

Adult wild-type zebrafish (*Danio rerio*) (0.5 g - 0.8 g) were kept at the experimental fish facilities at SRMIST. They were acclimatized in a 160 L aquarium with freshwater (pH 7.4, conductance 550 μ S) equipped with Eheim-biofilters and aerated by oxygen stones for one week prior to experimentation. Fish were provided with commercial feed twice daily and maintained at a 14 h light and 10 h dark cycle in a thermostat-controlled room (28.5°C \pm 0.5°C).

DRUG TREATMENT:

[13]. Zebrafish (*Danio rerio*) larvae have emerged as a useful tool to study IBD and gastrointestinal diseases.

[14,15]. Compared with humans, the zebrafish has a highly similar gastrointestinal system, with a liver, gall bladder, pancreas, and intestinal tract with comparable absorptive and secretory functions. In addition, a genome alignment showed that zebrafish orthologs exist for over 70% of human genes and showed a susceptibility to mutagenesis and the availability of transgenic technology.

[16,17]. *NOD1* and *NOD2* encode two types of nucleotide-binding oligomerization domain-containing proteins, both of which recognize bacterial molecules and stimulate an immune reaction.

[18]. In addition to *n*-ethyl-*n*-nitrosourea ENU, intestinal damage models are often induced by dextran sodium sulphate (DSS) or trinitrobenzene sulfonic acid (TNBS).

[19]. Chemical models of enterocolitis are induced conveniently by oxazolone, lipopolysaccharide (LPS), and glafenine. Chemical genetic methods can also manipulate the host response to injury, which is convenient for use in IBD models.

PHYTOCHEMICAL ANALYSIS

It is used to determine the presence of phytochemicals in the polyherbal extract. Poly herbals were collected from a local area. These poly herbals were washed and dried under shade, weighed equally (25g) and are powdered to a coarse form. The coarse particles are kept in a beaker containing ethanol and distilled water in a ratio of 70:30. The beaker is shaken for every two hours. The supernatant was filtered with a help of a Whatman filter paper. The filtrate was collected and carried out to perform out the various phytochemical tests. Mayer's test, Dragendroff's test, Hager's test, Wagner's test was performed to determine the presence of alkaloids. Fehling's test, Benedict's test, Barfoed's test was performed to determine the presence of carbohydrates. Biuret's test, Millon's test was performed to determine the presence of Proteins. Similarly, test for steroids, sterols, phenols, tannins, flavonoids, gums and mucilage, glycosides, saponins, terpenes were carried out to detect the presence of respective phytoconstituents.

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR):

[20]. It is the most powerful tool for identifying the types of chemical bonds or functional groups present in the phytochemicals. The wave length of light absorbed and the salient feature of the chemical bonds can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a compound can be determined.

The test sample was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample disc. The sample was loaded in FTIR spectroscope (SRMIST, Chennai,) with a scan range from 400 to 4000cm⁻¹ with a resolution of 2cm⁻¹.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY:

[21]. HPTLC fingerprinting analysis, 2 µL of the test solution and 2 µL of the test solution and 2 µL of standard solution were loaded as 5 mm band length in the 3 and 10 Silica gel 60F₂₅₄ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The sample's loaded plate was kept in TLC twin trough developing chamber with respective mobile phase and the plate was developed in the respective mobile phase 90 mm. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254 nm. The developed plate was sprayed with respective spray reagent and dried at 60° C in hot air oven. The plate was photo documented at daylight and UV 366 nm mode using photo documentation (CAMAG REPROSTAR 3) chamber. After derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at 254 nm. The peak table, peak display and peak densitogram were noted.

III. SCREENING METHODS ANTIOXIDANT ACTIVITY BY DPPH RADICAL SCAVENGING METHOD:

[22,23]. The free radical scavenging activity of different concentrations of poly herbal extract were measured by DPPH. In brief, 0.1 mM solution of DPPH in ethanol was prepared. This solution (1 ml) was added to 3 ml of different concentrations of a poly herbal extract in ethanol at different concentration (5, 10, 15, 20, 25 µg/ml). The mixture was shaken vigorously and allowed to stand at room temp for 30 minutes. Then, absorbance was measured at 516 nm by using

spectrophotometer (UV-VIS Shimadzu). Reference standard compound being used was ascorbic acid and experiment was done. The IC₅₀ value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using Log dose inhibition curve. The Lower absorbance of the reaction mixture indicated higher free radical activity. The percent DPPH scavenging effect was calculated by using following equation:

DPPH scavenging effect (%) or percent inhibition = $A_0 - A_1 / A_0 \times 100$

Where A₀ was the absorbance of control reaction and A₁ was the absorbance in presence of test or standard sample.

% DPPH radical scavenging:

Absorbance of standard- Test sample
----- *100
Absorbance of standard

DSS INDUCED COLITIS IN ZEBRAFISH MODEL-HOUSING CONDITIONS:

[24]. Adult wild-type zebrafish (*Danio rerio*) (0.5 g - 0.8 g) were kept at the experimental fish facilities at SRMIST, Chennai. They were acclimatized in a 160 L aquarium with freshwater (pH 7.4, conductance 550 µS) equipped with Eheimbio-filters and aerated by oxygen stones for one week prior to experimentation. Fish were provided with commercial feed, twice daily and maintained at a 14 h light and 10 h dark cycle in a thermostat-controlled room (28.5°C ± 0.5°C).

DSS induced colitis: Twenty-four hours before the experimental procedure, groups of three were placed in 1L tanks and feeding was stopped. For induction of colitis, 12 fish (3 × 4) were anaesthetized Ice cubes. 0.25% of DSS induction to zebrafish for 7days. Control group, DSS induced, DSS + low dose (250µg/ml), DSS + High dose (500µg/ml). Seventh day submitted to histopathological evaluation.

S.No	PHYTOCHEMICAL TEST	ETHANOLIC
1	Test for Alkaloids	+
2	Test for Protein	-
3	Test for Sterols	-
4	Test for Tannins	+
5	Test for Flavonoids	+
6	Test for Phenols	-
7	Test for Carbohydrates	+
8	Test for Saponins	-
9	Test for Glycosides	+
10	Test for Triterpenoids	+
11	Test for Cholesterol	+

Wave number in cm-1		Vibrational Assignment- Functional groups
S.no	Peak	
1.	3312.63	O-H-Stretching- Amies, Carboxylic Acid, Alcohols, Alkenes
2.	2934.96	C-H stretching- Alkanes, Alkyls, Carboxylic Acid
3.	1594.36	N-H bending- Amide, Amines
4.	1270.19	C-F stretching- Alkyl halides, ester
5.	1033.76	C-F Stretching-Alkyl halides, Alcohols, Ester
6.	1033.76	C-H bending- Aromatic Compounds, Alkenes
7.	819	=C-H bending- Alkenes
8.	773.14	=C-H bending- Alkenes, Aromatic compounds
9.	709.74	C=H bending Amines
10.	537.07	C-Br stretching- Alkyl halides

Analysis of FTIR

IV. RESULTS

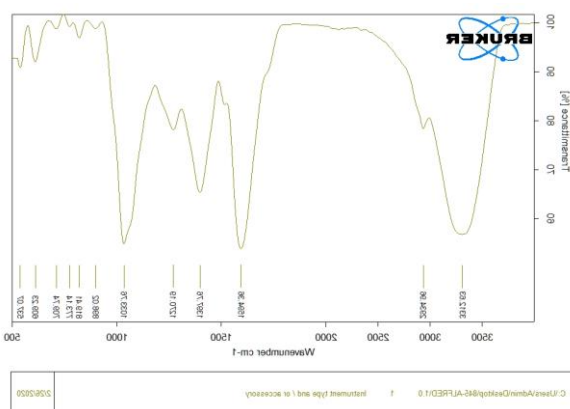
PHYTOCHEMICAL SCREENING:

PHYTOCHEMICAL REPORT OF POLY HERBAL EXTRACT

The phytochemical analysis revealed the presence of carbohydrates, phenols, proteins triterpenoids, flavonoids, and glycosides the data were tabulated below.

FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

FT-IR spectroscopy can provide strong insight into the structural and functional alterations induced by various factors due to its high sensitivity. FT-IR technique was used for assessment the type of functional groups,

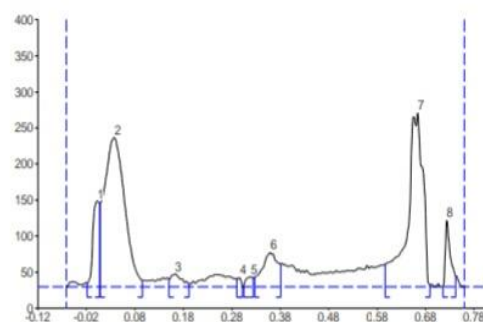


FTIR assay with functional groups

The present study, the FTIR spectroscopic analysis showed the presence of phytoconstituents in Polyherbal extract. The preliminary phytochemical screening of poly herbal extract showed (Figure:3) the presence of functional groups in phytochemicals screened such as **Alkaloids, Carbohydrates, Flavonoids, Tannins and Triterpenoids.**

Flavonoids and Triterpenoids present in this extract responsible for protective effect of inflammation in colitis.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY:



HPTLC

HTPLC Peaks Shows Phytoconstituents present in the polyherbal extract which responsible for decreases the intestinal inflammation.

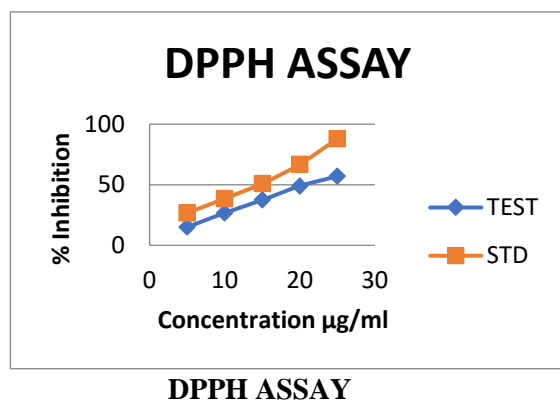


Different groups of Zebrafish DSS induced colitis

ANTIOXIDANT ACTIVITY-DPPH ASSAY

DPPH is stable free radical at room temperature and accepts an electron / hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical is determined by the decrease in its absorbance at 516 nm, induced by antioxidants. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and radicals, progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity. The results indicate that the

extract reduces the radicals to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principle. DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up. The results of DPPH assay have been tabulated.

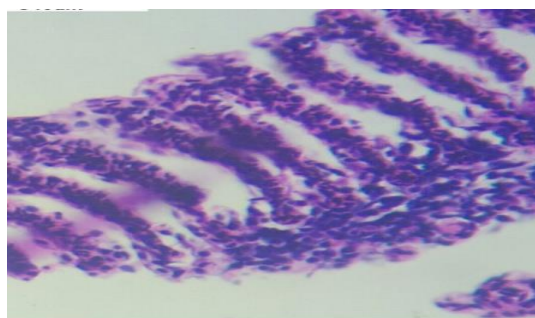


The DPPH radical scavenging capacity of poly herbal extract and standard drug (Ascorbic acid) were shown in Table 6. The percentages of DPPH scavenging activity of poly herbal found to be 45.86, 57.83, 66.82, 74.81, 82.65 % at the concentrations of 5, 10, 15, 20, 25 mg/ml respectively. The highest percentage of inhibition was exhibited 82.65% at the highest concentration.

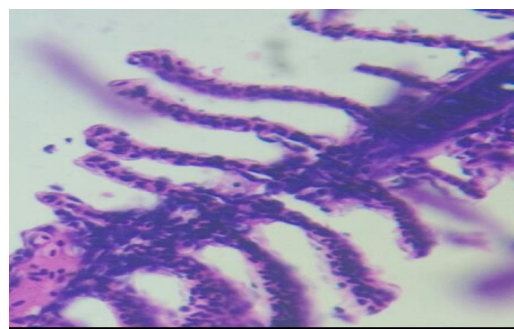
Concentration (mg/ml)	% Inhibition of test	% Inhibition of standard
5	14.96	26.65
10	26.68	38.58
15	37.37	50.96
20	48.87	66.94
25	56.94	87.87

DSS INDUCED COLITIS:

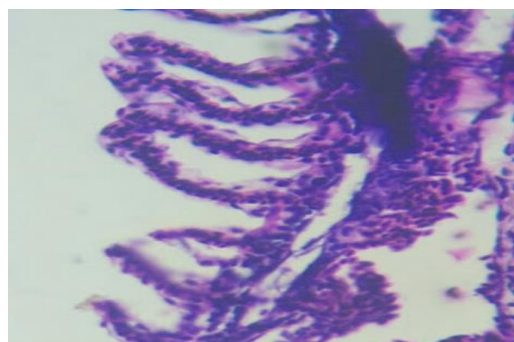
Control group shows normal intestinal layer, DSS induced colitis severe ulceration in intestinal epithelial cells, Low dose and high dose of Poly herbal extract shows decrease in intestinal inflammation.



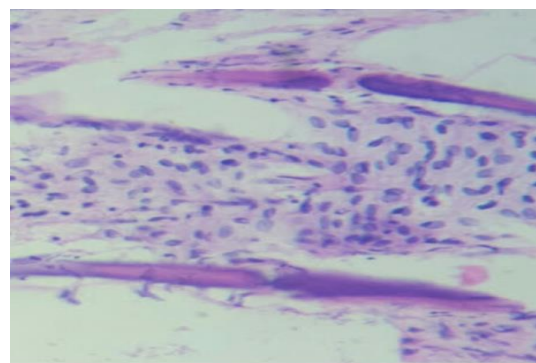
CONTROL



LOW-DOSE



HIGH-DOSE



DSS-INDUCED

Histopathological slides

Histopathological slides show control normal intestinal cells, DSS treated rupture of intestinal epithelial cells, Low dose (250µg/ml) and High dose (500µg/ml) represents the decrease the intestinal inflammation.

V. DISCUSSION

In present study, Dextran sodium sulphate was used to induce inflammatory bowel disease in Zebrafish. In DSS induction method of colitis, single dose is sufficient to induce IBD. This model resembles to IBD in human beings in way of pathogenesis, histopathological features and inflammatory mediator profile. The distinctive features of induced colitis using DSS in Zebrafish is actually an imbalance between oxidant and antioxidant substances. It was reported that infiltration of neutrophils leads to formation of superoxide anion and initiation of a cascade for the

formation of various reactive species. This may lead to the development of tissue necrosis and mucosal dysfunction.

World health organization (WHO) guidelines and procedures are essential for increasing herbal products in market. The phytoconstituents present in the Polyherbal extract exhibits a substantial reduction of intestinal inflammation and Alteration in goblet cells.

VI. CONCLUSION

There are several hypotheses on cause of inflammatory bowel disease. Which occurs both in men and women, however there is limited information regarding the mechanism of its development. The therapeutic approaches to IBD are mainly symptomatic since very less information are available on the disease.

Few agents such as Amino salicylates, Corticosteroids, Immunomodulators, Antibiotics are preferred both for human and animals in IBD treatment. However due to several adverse effect caused by synthetic drugs the plant-based remedies are preferable in IBD treatment.

The present work aimed in the **Anti-Ulcerative effect of Polyherbal extract in Zebrafish model.**

The present study revealed that the Polyherbal extract presence of Phytoconstituents such as flavonoids, Triterpenoids, Alkaloids, Carbohydrates, Tannins were identified by preliminary phytochemical screening, and the functional groups are identified FTIR. The functional groups present in this extract such as **C-Br, C-Cl, CH₂=CH₂, C-X, OH, R-OR, R-COOCR, R-NH₂, C-F, C-I, C₆H₅R, R-CCOH.** This resembles the functional group present in the flavonoids and Triterpenoids.

VII. ACKNOWLEDGEMENT:

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