UPREGULATION OF CYCLOOXYGENASE SYSTEM AND GROWTH FACTORS AS PLAUSABLE MECHANISM OF ANTINUCLEROGENIC ACTIVITY OF LEAF OF *PIPER BETLE* Linn: A MOLECULAR INSIGHT

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Abstract:

Allylpyrocatechol, the active component obtained from ethanol extract of leaves of Piper betle Linn *at a dose of 120mg/kg body weight was shown to be an antiulcerogenic agent . Allylpyrocatechol was isolated from PBE by column chromatographic separation followed by preparative TLC and tested in rat ulcer model. Histological studies reveal that damage in the mucosal epithelium was corrected and the disrupted epithelium (as seen in ulcer) reversed with the test compound. The cellular, molecular and biochemical factors underlying the healing of gastric ulcer, such as, growth factors (EGF, EGF-R, SMAD-4) and cyclooxygenases (COX-1 and COX-2) were observed to be up-regulated in presence of APC during healing process. All the data showed statistical significance (by one way ANOVA) when compared to the experimental control value.*

Key words: *Allylpyrocatechol, COX-1,2; EGF/EGF-R, Gastric ulcer, Piper betle Linn.*

Introduction:

Gastric ulcer is a disease, very common worldwide. Apart from the major causative agent, *H.pylori*, the spiral bacteria, the next common cause of induction of gastric ulcer are the NSAIDs (Non-steroidal anti-inflammatory drugs), which are very commonly taken by the people as pain-killers. The NSAIDs induce ulcer by several mechanisms, one being the involvement of free radicals by neutrophil activation. The free radical damage occurs mainly by the lipid peroxidation. There has been an accumulative evidence of the involvement of prostaglandin, cyclooxygenase 1 & 2, Nitric oxide, inducible Nitric oxide synthatase and different cytokines are induced or increased by gastric ulceration and might also contribute ulcer healing. There is a cross-over role of COX-1 and COX-2 with inflammatory process and ulcer healing. It is reported that several growth factors, when up- regulated, enhance the healing process of ulcer. The most widely involved growth factors being Epidermal Growth Factor (EGF), its receptor (EGF-R) and the components of TGF-pathway (eg. SMAD-4). Numbers of synthetic drugs are available in the market and are effectively used to cure NSAID-induced gastric ulcer, but all of them are reported to have several side effects. The most widely used drug, being, Misoprostol, is reported to produce diarrhea, nausea, vomiting etc. It's even not permissible for pregnant women, as Misoprostol has an abortive property. Hence, the need for a safer drug to combat against NSAID-induced gastric ulcer is yet not fulfilled that limits the usage of NSAID. Plant drugs are normally known to have lesser or no side-effects. The plant-parts,

which are already edible to people are even expected to be non-toxic. Hence, the ultimate finding of a drug from plant origin, which can heal the NSAID-induced gastric ulcer, enhance the COX iso-forms and the express the up-regulation of the Growth factors can lead to the development of a drug with better prospect and accessible to the common people.

Materials and Methods:

Extraction of Plant part in Suitable Solvent:

Fresh leaves (P. betle) 250 gm was dissolved in 1L of 95% alcohol or 1L of Ethyl Alcohol (as specified before) at 4oC x 7 days with change and intermittent shaking. Filter through nylon cloth. The pooled filtrate is put in the rotary evaporator under vacuum and the extract, devoid of organic solvent was isolated. It was lyophilised under pressure, to remove water content and stored at vacuum (PBE= P. betle Ethanolic extract)

Isolation of compounds from PBE:

1.71 g of PBE and 50ml ethanol was filtered and the filtrate treated with activated charcoal(0.1g)and allowed to stand overnight(to get rid of the chlorophyll pigment, filtered, concentrated in vacuum , when a dark brown sticky mass was obtained.(1.038g) (hence, 60.7% yield).0.5g of this was subjected to column chromatography over a silica gel column containing 5g of silica gel and eluted with 10% methanol-chloroform and 5 fractions were collected (~50ml each).

Fraction 1-Concentrated in vacuum and put to preparative TLC on a silica gel plate, using 5% EtOAc-Hexane as the solvent. The TLC band at Rf 0.7 was scrapped from the plate and eluted with chloroform. Concentration in vacuum furnished a pure compound (8.4mg), which was identified as Chevibetol by IR and 1H NMR Spectroscopy. (The compound was already reported.)

Fraction 2- Concentrated in vacuum and subjected to preparative TLC using 5% methanol- chloroform as solvent. It showed a major band with a polymeric material which gave colouration with FeCl3 spary, indicating its polyphenolic nature. Multiple developments of the TLC plate using same solvent resolved the major band into 2 closely spaced bands with Rf 0.3 and 0.35 respectively. They were scrapped and eluted with 20% methanol-chloroform. On concentration 2 compounds were obtained (49.2mg and 25.3mg respectively). The former was found to be Chevicol or Allylpyrocatechol, whereas, the latter, was shown to be unstable and gets oxidized very quickly. Spectral data showed that this compound was not reported earlier.

Fraction 4 and 5- on concentration gave 340.8mg and 73.0mg residues respectively. Positive Molisch test showed their Glycosidic nature. They didn't show any antioxidant property, Hence, no further study with them was done. Allylpyrocatechol was designated as P1 in our study.

Preparation of Drug from Different Extract:

The dose of the extracts to be given to the Rats of the experimental ulcer model, are standardized. The different standardized dosages are: PBE= 120mg/kg BW. The active fractions are given at a dose just similar to their percentage yields from the extracts. Hence the dose of P1 is selected as followed: P1=2.0 mg/kg BW.
All of them are mixed with 2% Gum Acacia, which works as a binder and reported to be a non-toxic one. The extracts are prepared in

distilled water as the solvent and are given by oral intubation to the Rats, using feeding cannulae 1 ml. of the prepared drugs are given each day.

Experimental Ulcer Model:

Under healing: Design of experiment: Rats: (Male Wister strain, male, wt 100-120 g.)

Group A	Group B	Group C	Group D
(5 Rats)	(5 Rats)	(15 Rats)	(15 Rats)

1ml of Vehicle (2% gum acacia) x 10 days, sacrificed (Normal control)

Indomethacin 30 mg/kg body wt. Overnight fasting, water adlibtium

Group B	Group C	Group D
(5 Rats)	(15 Rats)	(15 Rats)
Fasting overnight Sacrificed 4Hours	without drug (extractive) only vehicle	Treated with Drugs (2%Gum in 2%Gum Acacia After Indomethacin Administration acacia, 1ml) At pre-determined doses.
(Experimental Control)	(Experimental) Sacrificed on 7th day	

Quantification of Mucosal Injury:

The area of mucosal damage was calculated in square millimeters and expressed as percentage of the glandular stomach according to Szabo et.al.

Preparation of Tissue Homogenate:

Rat: Stomach tissue after the weight was homogenized using a glass Teflon homogenizing tube in 50mM phosphate Saline Buffer, pH 7.2 under cold condition. The homogenate was centrifuged at 2000 rpm for 10min and the supernatant, collected for Biochemical analysis.

Total Protein:

Total protein is estimated by the method of Lowry et al. Briefly, samples and standards (1mg/ml BSA in double distilled water) in different tubes are treated with 5ml of RA mixture (4.8% Na-K-Tartarate, 2% CuSO4 and 3% Na2CO3 in 0.1N NaOH at a ratio of 1:1:48 by volume). Then Phenol regent (half diluted double distilled water) is added to the reaction mixture with continual vortexing. The reaction mixture is allowed to stand for 30 minutes at room temperature and optical density is measured at 710nm using water as reagent blank.

Estimation of Lipid Peroxidation:

Lipid peroxidation in the ulcer tissue was measured by quantification of the Thio-barbituric acid reactive substances produced in the tissue. The method is as discussed earlier (261).

Histology of Gastric Mucosal Tissue:

Histopathological examination of gastric tissue was done as per the method described by Bancroft et al (275). The gastric tissue was immediately fixed in 10% buffered formal saline for 24 hours.

1. Tissue was washed in tap water.
2. Kept in 70% alcohol for 24 hours
3. Transferred to 90% alcohol for 24 hours.
4. Dehydrated in absolute alcohol- 3 changes for ½ hour each.
5. Put in Xylol for clearing- 3 changes for 15 minutes each.
6. Put in molten paraffin (fresh) (60°C-62°C).
7. Blocked by fresh molten paraffin. Each block was numbered.
8. The sections were cut with a Microtome with a thickness of 3-5 cm.
9. The ribbons of sections were floated Immediately in slides containing a drop of warm distilled water and the wrinkles were removed by teasing with a needle. Slides were placed on hot plate, so that the water evaporates, paraffin melts and the sections get fixed on the slides.

HaematoxylinE and Eosin Staining Solutions:

Harris' Method (Harris' Alum Haematoxylene):

a) Haematoxylene: Haematoxylene (5g); Absolute Alcohol (50cc); Ammonium/Potassium Alum (100g); Distilled Water(1000cc); Mercuric Oxide (2.5g); Glacial Acetic Acid (20cc);100g of Potassium Alum was dissolved in 100cc of warm Distilled water. 5g of Haematoxylene was dissolved in 50cc Absolute Alcohol. This mixture was added to the first mixture and brought to boil rapidly. Then 2.5g of mercuric oxide was added. The whole mixture was cooled rapidly by plunging the flask into cold

water. When the solution was cooled, 20 ml of Glacial acetic acid was added. The stain was filtered before using.

b) Eosin: Eosin Y (2mg); Distilled Water (100ml); 1mg of Eosin Y was dissolved in 100ml of distilled water. Thymol was added to inhibit fungal growth.

Procedure:

The sections were de-paraffinized after warming on hot tray for 2-3 minutes. The slides were put in Xylene for 20 minutes. They were hydrated through different grades of alcohol (100%, 90%, 70%, 50%, and 30%). Finally; they were kept in distilled water for 10 minutes. Haematoxylene stain was added and kept for 5 minutes. Slides were washed under running tap water for 15 minutes. Slides were passed through Alcohol gradation (30%, 50%, and 70%).Eosin was added and kept for 2 minutes. Slides were kept in 90% alcohol for 5 minutes. Slides were kept in 100% alcohol for 5 minutes and finally they were kept in Xylene for 30 minutes. Mounted in DPX.

Results: Nuclei Blue Black; Cytoplasm varying shades of Pink.

Estimation of COX-1 and –2, i-NOS, IL-8RA (CXCR1), Epidermal Growth Factor and Smad- 4 Protein:

Immunohistochemistry:

Mucosal specimen were deparaffinized in Xylene followed by 100% Alcohol. Kept in Methanol+H2O2 solution (200:1) for ½ hour. Washed in running tap water. Kept in PBS(pH 7.6) for 10 minutes. Slides were wiped and incubated with Blocking solution (Normal Rabbit Serum, 1:5 in PBS).Incubated with Primary antibody [Cox-1(1:50); Cox-2,IL-8RA, Smad-4, EGF-R(1:100) in PBS] for 1 hour. Washed in PBS. Incubated with secondary antibody HRP-labeled [1:500 anti-mouse IgG (for Cox-1, Cox-2, Smad-4); 1:500 anti-goat IgG (for IL-8RA); 1:500 anti-sheep IgG (for EGF-R)] for 1 hour. Washed in PBS.DAB (Diamino benzidine) solution was added and kept for 40 seconds. Washed in running water. Counterstained with Haematoxylene for 2 minutes. Washed in running water. Kept in 100% alcohol for 30 minutes. Kept in Xylene for overnight. Mounted in DPX.

Immunofluorescence:

Mucosal specimen were deparaffinized in Xylene followed by 100% Alcohol. Kept in 4% p-Formaldehyed. Washed in PBS (pH 7.6) for 10 minutes. Kept in Triton-X for 5 minutes. Slides were washed and incubated with Blocking solution (Normal Rabbit Serum, 1:5 in PBS).Incubated with Primary antibody [EGF-R(1:100) in PBS] for 1 hour. Washed in PBS. Incubated with secondary antibody FITC-labeled [1:500 anti-sheep IgG] for 1 hour. Washed in PBS. Mounted in DPX.

Polymerase Chain reaction:

RNA extraction and RT-PCR analysis: Total RNA was isolated by TRIzol method (Invitrogen) according to the manufacturer's protocol. 1 □g of RNA treated with RNase-OUT ribonuclease inhibitor (Invitrogen) was used for cDNA synthesis. Reverse-transcription using Superscript reverse transcriptase-II (Invitrogen) and Oligo dT (Invitrogen), to prime the reaction was carried out. PCR primers were selected to distinguish between cDNA and genomic DNA by using individual primers specific for different exons. 1 □l of cDNA was amplified by polymerase chain reaction

using Abgene 2X PCR master mix (Abgene, UK) and appropriate primers (Refer table M2). The expression of genes such as EGF and SMAD-4were checked. For all the genes, PCR were performed for 35 cycles, consisting of an initial denaturation at 94o C for 1 min, then 94o C for 30 sec, annealing temperature of the respective gene primer for 45 sec, 72o C for 1 min and was terminated by final extension at 72o C for 5 minutes.

Table 1: sequences of primers used in RT-PCR analysis.

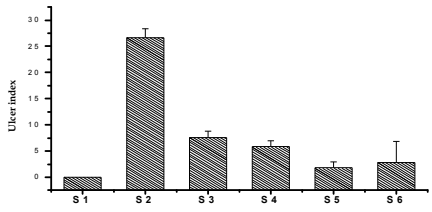
Genes	Primer Sequences
EGFR-F	AAGGATGTGAAGTGTGG
EGFR-R	ACTTTCTCACCTTCTGG
Smad4-F	AAGGTGGGGAAAGTGAAAC
Smad4-R	ATGCTTTAGTTCATTCTTGTG

Statistical tests by ANOVA.

The data generated from the different set of experiments were evaluated statistically using ANOVA, to determine the significance of Mean+SEM values. Differences were considered significant at $p<0.05$.

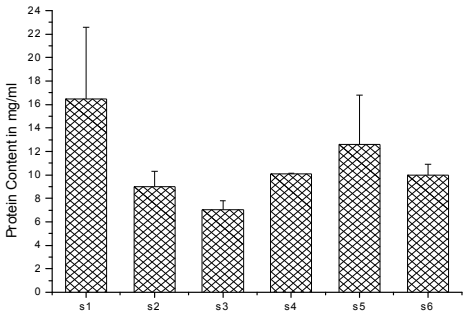
Results:

1. Ulcer index:



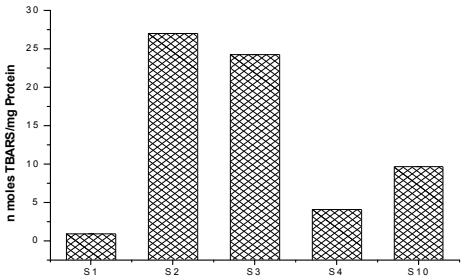
S1= Normal Rat Stomach; S2= 0th Day Ulcer Rat Stomach; S3= 7th Day Ulcer Rat Stomach (without treatment) S4= 7th Day Ulcer Rat Stomach (treated with PBE) ;S5= 7th Day Ulcer Rat Stomach (treated with P1) S6= 7th Day Ulcer Rat Stomach (treated with Misoprostol)

2. Total Protein



S1= Normal Rat Stomach ; S2= 0th Day Ulcer Rat Stomach ; S3= 7th Day Ulcer Rat Stomach (without treatment) S4= 7th Day Ulcer Rat Stomach (treated with PBE) ; S5= 7th Day Ulcer Rat Stomach (treated with P1) S6= 7th Day Ulcer Rat Stomach (treated with Misoprostol)

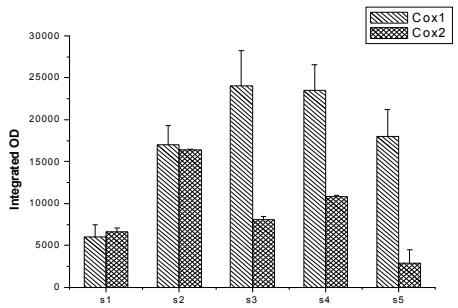
3. Lipid Peroxidation



S1= Normal Rat Stomach;S2= 0th Day Ulcer Rat Stomach;S3= 7th Day Ulcer Rat Stomach(without treatment) S4= 7th Day Ulcer Rat Stomach (treated with P1); S5=7th Day Ulcer Rat Stomach (treated with Misoprostol)

4. Immunohistochemistry of COX-1 and COX-2

COX-1 and COX-2 are two important factors in the induction of gastric ulcer. Though controversial, but generally it is known that COX-1 gets suppressed by the non-selective NSAIDs, forming gastric ulcer. COX-2 also gets lowered down. But in case of healing COX-1 level increases along with COX-2.



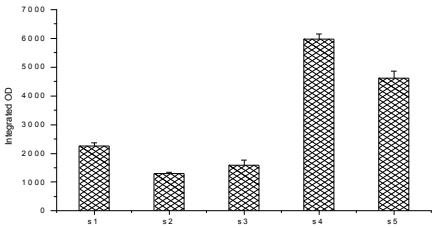
S1= Normal Rat Stomach;S2= 0th Day Ulcer Rat Stomach;S3= 7th Day Ulcer Rat Stomach (without treatment) S4= 7th Day Ulcer Rat Stomach (treated with P1); S5=7th Day Ulcer Rat Stomach (treated with Misoprostol)

Immunohistological data showed that COX-2 level lowers down at the onset of ulcer induction. In the 7th day ulcerated tissue, the COX-2 gets increased but the COX-1 still remains suppressed, showing ulcerated condition, as it is known that suppression of COX-1 leads to ulcer formation. But with the extracts the COX-1 as well as COX-2 level rises. The increase in COX-1 level is much more than the rise in COX-2. The results showed that P1 is effective in increasing the level of both COX-1 and COX-2.

5.Estimation of EGF, EGF-R:

Epidermal Growth Factor is an important factor in promoting the healing effect of gastric ulcer. In a normal tissue this protein is not very significant in amount but, whenever there is an ulcer formation, the protein gets synthesized in a large amount. A good healing agent should promote the synthesis of this protein as it helps in cell division and growth of new healthy cells which replace the disrupted ones.

a. By ELISA: ELISA is a standard method to measure the binding of antibody to EGF antigen. It is quick and easy method, hence, the binding of anti-EGF antibody can prove the presence of EGF in the tissue sample.

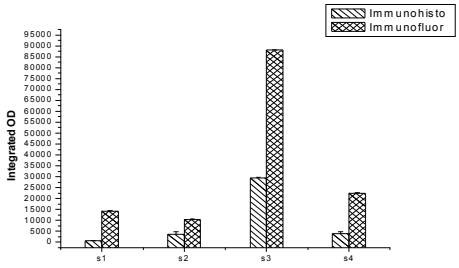


The ELISA shows large amount of EGF in the 0th day ulcerated tissue but without any treatment the 7th day ulcer tissue showed a decrease in the EGF amount, whereas, PI showed increase in the EGF amount, suggesting the quick healing procedure, as EGF enhances the healing.

b) Estimation of EGF-R by Immunohistochemistry and Immunofluorescence:

Since, Immunohistochemistry and Immunofluorescence are two important method in showing the binding of EGF-R molecule in the gastric epidermis, they are done and the colour developed by them are measured by a software, Biovis, which measures the amount of binding by measuring the Integrated OD against the binding in percentage area of the slide. The rise in Integrated OD is a measure of high

binding of the antibody.



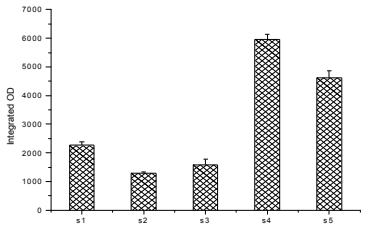
S1= 0th Day Ulcer Rat Stomach;S2= 7th Day Ulcer Rat Stomach(without treatment);S3= 7th Day Ulcer Rat Stomach (treated with P1) S4= 7th Day Ulcer Rat Stomach (treated with Misoprostol);

c) Estimation of EGF-R and Smad-4 by RT-PCR:

The expressions of genes were studied through PCR analysis. After the experiment DNA bands were obtained through Agarose gel electrophoresis. The result was documented by Gel Documentation instrument. The bands showed that P1 treated gastric tissue expressed EGF-R and Smad-4 gene. Misoprostol expressed EGF-R to a lesser content. On the onset of ulcer (U0) Smad-4 showed a faint band.

6. Estimation of SMAD-4 by Immunohistochemistry:

SMAD-4 protein is a component of the TGF-□ pathway, which is known to increase the healing of gastric ulcer or any other tissue damage. The TGF□ pathway in turn is related with the EGF regulation pathway, so activation of SMAD-4 refers to the activation of EGF regulation pathway also.



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Smad-4 level was decreased in the ulcer 0th day ulcer tissue, which increased in the 7th day ulcer tissue. But the treated groups showed a good increase in the smad-4 expression. P1 proved to be the most effective in increasing the expression of Smad-4, whereas, the Misoprostol and E4 also showed a significant increase.

Discussion:

Ethanolic extract of leaves of Piper betle was shown from the similar laboratory to exhibit significant healing effect on the NSAID-induced gastric ulcer as evident from various biochemical parameters.(ref.). It was also shown in our previous paper that Ulcer formation , induced by Indomethacin, an NSAID, is related to the inhibition of the cyclooxygenase, that prevents the prostaglandin biosynthesis and in turn inhibits the release of the defensive factors of the stomach, mucin. The ulcer index showed that PBE gives 78.28% protection if 0th day ulcer is taken as 100% ulcer. Whereas,P1 gives 93.41% protection; and synthetic drug gives 85.39% protection.

Results show that treatment with the extracts significantly decrease the level of lipid peroxidation product, TBARS in gastric

tissue compared to ulcerated rats (fig 17 and table6). The decrease in the peroxidation level of lipid indicates a plausible mechanism of free radical involvement. The peroxidised lipids are the products of ROS damage on the cell membrane leading to disintegration and de-structuring of the fluid dynamic mosaic model, leading to cell death and formation of ulcer.

NSAIDs are known to induce ulcers during the course of their anti-inflammatory action by prostaglandin synthatase inhibition (through cyclooxygenase pathway). Immunohistological data showed that COX-1 and COX-2 comes to a level after a depression in cox-1 and slight increase in cox-2 level by the induction of NSAID, in the ulcerated model. On the 7th day both the COX-1 and 2 level increases. But with the extracts the cox-1 level rises much than the rise in COX-2.Recently it has been found that various gastric stressors (NSAIDs, alcohol, oxidative stressors, and acids) decrease the number of viable gastric pit cells by inducing both apoptosis and necrosis.(295,296) Thus, increasing the number of gastric pit cells could provide a new target for anti-ulcer drugs. In fact, growth factors for gastric mucosa were shown to be effective in combating ulcers in rats by increasing the number of gastric pit cells present (297). Hence activation of SMAD group of proteins could provide the basis of new types of anti-ulcer drugs, which increase the number of gastric pit cells by inhibiting their spontaneous apoptosis and stimulating their cell proliferation.

PCR is the best method to see the expression of certain genes in any tissue. In our experiment, EGF and SMAD protein were over- expressed in the P1 tissue, which is a direct proof of their up-regulation during the healing by these components. The present investigation thus emphasizes on the basic and fundamental research, detailing the effect of P1 in eradicating gastric ulcer. The plausible mechanism of P1behind the healing activity can be many, including their antioxidant property, immunomodulatory property, growth factor promoting property as well as COX up-regulating property. Our study explores the possibility for the development of some new drugs from plant source that become all the more relevant for an economically weak country, like India, which is rich in natural resources. The study is based on the fact that the ethanolic extracts of Piper betle leaves accelerate the healing of gastric ulcer induced by Indomethacin. The importance of the present study multiplies as the active components of it, which is a simple molecule like allylpyrocatechol, is proved to be a very good agent in healing gastric ulcer.

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Abbreviaion Used:

APC: Allyl pyrocatechol (P1) ; COX: Cyclooxygenase; EGF: Epidermal Growth Factor; EGF-R: Epidermal Growth Factor-Receptor; MDA: Malondialdehyde; PBE: Piper betle; Linn Ethanolic extract; TBARS: Thio-barbituric acid reactive substances

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