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Effect of *Pleurotus ostreatus* Extract on Epidermal Growth Factor Receptor Expression during Healing of Aspirin-induced Peptic Ulcer in Male Rats

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Abstract

Peptic ulcer is an international problem in the last two centuries, including gastric and duodenal ulcers. The great majority of peptic ulcer disease is due to peptic inflammation caused by the infection of *Helicobacter pylori* and non-steroidal anti-inflammatory medicines. The purpose of this study was to investigate the healing role of *P.ostreatus* extract on peptic ulcers throughout the detection effect of *P. ostreatus* extract on epidermal growth factor receptor (EGFR) expression intensity during ulcer healing. In this study, 60 male rats of *Rattus norvegicus* were used; rats were divided into negative control group (C) given standard diet and distilled water only along study period; the remaining rats were treated by aspirin (100 mg/kg body weight) for induction of ulcer by oral dosage for one week, then these animals were divided into T1 (positive control group or aspirin ulcerated group which developed peptic ulcer through oral dosage for one week and remained without treatment for 20 day), T2 (group treated with omeprazole for 20 days), T3 (group treated with alcoholic extract of *P.ostreatus* for 20 days), T4 (group treated with polysaccharide of *P. ostreatus* for 20 days) and T5 (group with chitin and chitosan of *P. ostreatus* for 20 days). The immunohistochemical technique was used to identify the gene expression of EGFR. The results of this study showed there is a significant increase in the mean of EGFR expression intensity in the stomach and duodenal ulcer treated by mushroom extract and omeprazole in comparison with the untreated ulcer in positive control group T1 ($p<0.05$) at 10 day and 20 days. Moreover, the highest means of expression of EGFR intensity were recorded in the case of T3 followed by the treatment T4 at 10 and 20 days respectively. In conclusion, alcoholic extract of *P. ostrateus* has a significant role in peptic ulcer healing throughout the increasing expression of EGFR intensity.

Keywords: *P.ostrateus*, EGFR, peptic ulcer, polysaccharide, chitin&chitosan.

1. INTRODUCTION

Peptic ulcers, including gastric and duodenal ulcers, have long been classified as one of the most common diseases affecting humans in general, particularly young people (Kim *et al*.,2014). *H. pylori* infection and nonsteroidal anti-inflammatory drugs became the major causes of peptic ulcer (Mohan, 2018). Aspirin is an effective non-steroidal anti-inflammatory drug (NSAID) used to treat rheumatoid arthritis and related diseases and avoid thrombotic cardiovascular diseases. A major problem is a gastric ulcer associated with aspirin use. (Yin *et al*., 2007)

The process of ulcer healing involves growth factors that appear to play a crucial role in the stimulation of reconstruction of damaged mucosal structures. Among these factors, the most relevant are epidermal growth factor (EGF) (Kangwan *et al*.,2014). EGF ،a simple 53-amino acid polypeptide, is a cell-surface protein primarily responsible for increased cell proliferation at the mucosa of the ulcer margin and re-epithelialization; hence, its receptors are expressed as gastric glands dilate and the lining epithelial cells de-differentiate. It is mainly produced in salivary glands but can also be found in duodenal and pancreatic juice (Alese *et al.*,2017).

Epidermal growth factor was implicated in ulcer protection and ulcer healing. Through interaction with its cell surface receptor (EGFR). studies found that EGFR showed good digestive tract mucosal protection. Its expression in normal gastric mucosa is few, while gastrointestinal damage can increase its level (Aupperlee *et al*.,2012). EGFR can be activated by the binding of its ligands, including EGF, transforming growth factor-α, heparin-binding EGF-like growth factor, amphiregulin, betacellulin and epiregulin (Berasain and Avila 2014)

The effects of these growth factors are pleiotropic, ranging from the induction of DNA synthesis and changes in cell adhesion and motility to the stimulation of the differentiated cell function. In particular, EGF as well as TGF-α play important roles in the proliferation and differentiation of mucosal cells in the gastrointestinal tract, including the stomach (Brandl *et al*., 2010). More than 50% of all modern clinical drugs for peptic ulcers are of natural product origin and play a major role in the pharmaceutical industry's drug development programs

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(Lee *et al*., 2015). Mushrooms have recently become attractive as a functional food and as a source for new drug production; it has been stated that the functions of the immune system are activated by polysaccharides from the fruiting body or mycelium from a wide variety of edible mushrooms, fungi, and bacteria. Polysaccharides, including β-glucans, are now widely recognized as modifications of biological responses (Wilbers *et al*., 2016; Padilha *et al*., 2009). Dilfy *et al*., (2020) showed that chemical analysis of *P.ostreatus* showed that this mushrooms are an important source of various amino acids, vitamins, sugars, and minerals that considered important to body functions. Patel *et al*., (2012) stated that *P. ostreatus* had multiple active metabolites used as an anti-(aging, cholesterol, hyperglycemia, hypotensive, inflammatory, immunodeficient, microbial, mutagenic and neoplastic), metabolites besides as a defense and immunomodulation agent, which acts as an antibodies and tumor for the prevention and treatment of various human conditions. Present study aimed to use mushroom extract of Iraq cultivated *P.ostreatus* as success treatment for peptic ulcer throughout detection of the therapeutic effect of this mushrooms on EGFR during ulcer healing.

2. MATERIALS AND METHODS

2.1. Preparation of mushroom extracts

The fresh fruiting bodies of *Pleurotus ostreatus* were supplied from the Ministry of Science and Technology-Directorate of Agricultural Research-Baghdad. The first step in the preparation of mushroom extracts is cutting fresh fruiting bodies of mushroom (*P. ostreatus*) with a sharp knife into small parts. The parts were well air-dried for one week and kept in a night oven at a temperature of 40 ℃ to prevent rot. The air-dried mushroom sample was blended into powder using a blender. The crushed biomass (100 g) was placed in 400 ml of absolute methanol and incubated for 48 hours at 200 rpm. And the temperature is 37 ℃. To remove the biomass, the suspension was filtered by Whatman filter paper No.2. The supernatant was concentrated under lower pressure in a rotary evaporator at 50 ℃. The results were kept at 4 ℃ in the dried biomass (Jedinak and Sliva, 2008). Extraction of polysaccharide was prepared according to the method of (Gaur *et al*.2016), and extraction of chitin &chitosan according to the method of (Erdogan *et al*.,2017).

2.2. 2.2 Aspirin preparation

Aspirin was used for the induction of gastrointestinal ulcers in all experimental animals except the negative control group. To obtain the required dose (100 mg/kg b.w), a solution was prepared by dissolving 1 g of aspirin powder in 100 ml of 5% Carboxymethylcellulose to be aspirin concentration 10 mg /ml and given 1 ml / 100 g b.w (Seo *et al*.,2012).

2.3. Omeprazole preparation

Omeprazole is a standard drug, utilized in the treatment of ulcers developed in animals. To obtain the required dose (20 mg/kg BW), a Stock solution of this drug was prepared via dissolving of Omeprazole (0.2 g) in 100 ml of distilled water and given 1 ml /100 g body weight (Cavallini *et al.*,2006).

2.4. Animals

In this study, 60 male rats of *Rattus norvegicus* were used; rats were divided into negative control group (C) which given the standard diet and distilled water only along study period. The remaining rats are treated by aspirin (100mg/kg body weight) for induction of ulcer by oral dosage for one week, then these animals were divided into T1(positive control group or aspirin ulcerated group which developed peptic ulcer through oral dosage for one week and remained without treatment for 10 and 20 days), T2 (group ulcerated males rats were treated with omeprazole), T3 (group ulcerated males rats were treated with alcoholic extract of *P. ostreatus* for 10 and 20 days), T4(group ulcerated males rats were treated with polysaccharide of *P.ostreatus* for 10 and 20 days) and T5 (group ulcerated males rats were treated with chitin and chitosan of *P. ostreatus* for 10 and 20 days). Each group was anesthetized after the end of 10 days and 20 days and samples were taken directly.

2.5. Immunohistochemistry for detection of EGFR protein in peptic ulcer

Immunohistochemistry is a technique used for detecting cellular or tissue components (antigens) through antigen-antibody interactions. EGFR antibody and ABC staining system (Poly-HRP Anti Rabbit/Mouse IgG detection) were provided by Elab science. The procedure of immunohistochemistry for detection EGFR intensity in stomach and duodenum tissue was performed according to company manufacture. When the binding happens between antigen and antibody (Ag-Ab), a colored histochemical reaction product can be examined by light microscopy. Quantification of the EGFR expression (mm2) area by using Image-J software the photographs of the stomach and duodenum were digitalized and converted to binary images through grayscale imaging (Khan, 2004)

*2.6. **Statistical analysis***

The statistical significance of differences between groups was evaluated using single-way ANOVA, and the $p<0.05$ value was considered to be important. The sum of all values is expressed as a standard deviation in the mean.

3. Results

The results of this study showed a significant increase in the intensity of epidermal growth factor receptor (EGFR) expression in the treated stomach and duodenal ulcer compared with the untreated ulcer in the positive control group (T1) ($p<0.05$) during experimental period (**Table 1).** The results showed that EGFR expression intensity in the positive control group (T1) was extremely weak immuno-reactive in both stomach and duodenum tissue as (355 ±16 and 288±42) at 10 days and (409±20 and 315±66) at 20 days respectively.

The results revealed a significant increase in the mean of epidermal growth factor receptor (EGFR) expression intensity in treated stomach ulcer, and the highest means of expression intensity were recorded in groups (T3, T2 and T4) which were (3841±201, 2688 ±114 and 2656±138 respectively) at 10 days period. The intensity of EGFR expression in these groups decreases at 20 days with means (2809±113, 2301±106.5 and 1889±120 respectively). The lowest means of EGFR expression

intensity in stomach ulcers were recorded in the case of T5 which were 1197±99.65 and 1343±100 at 10 and 20 days respectively.

Statistically, all treatments (T2, T3, T4, T5) differed significantly in the mean of expression intensity of epidermal growth factor receptor (EGFR) when compared with the negative (C) and positive control group (T1) and in EGFR expression intensity between stomach and duodenum tissue at 10 and 20 day (**Table 2).**

Table 1. Effect mushroom extract and compounds on EGFR expression of gastrointestinal ulcer at 10 days and 20 day

Experimental Groups	Stomach EGFR expression intensity		Duodenal EGFR expression intensity	
	at 10 day	at 20 day	at 10 day	at 20 day
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
C	306±19	310±33.7	302.±27.8	336±28
T1	355±16	409±20	288±42	315±66
T2	2688±114**	2301±106.5**	656±58**	641±23.4**
T3	3841±201**	2809±113**	690±49.5**	668±30**
T4	2656±138**	1889±120**	567±17.5**	413±11**
T5	1197±99.65**	1343±100**	333±24.3**	403±19.8**

- SD= Standard Deviation; **= significant association in compared with T1 (P<0.05)

Table 2. Compared EGFR expression between treated stomach ulcer and treated duodenal ulcer at 10 days and 20 day

Experimental Groups	at 10 day			at 20 day		
	Stomach EGFR intensity	Duodenal EGFR intensity	P-value	Stomach EGFR intensity	Duodenal EGFR intensity	P-value
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
C	306±19	302.±27.8	0.436	310±33.7	336±28	0.711
T1	355±16	288±42	0.054	409±20	315±66	0.651
T2	2688±114**	656±58**	<0.0001[S]	2301±106.5**	641±23.4**	<0.0001[S]
T3	3841±201**	690±49.5**	<0.0001[S]	2809±113**	668±30**	<0.0001[S]
T4	2656±138**	567±17.5**	<0.0001[S]	1889±120**	413±11**	<0.0001[S]
T5	1197±99.65**	333±24.3**	<0.0001[S]	1343±100**	403±19.8**	<0.0001[S]

- SD= Standard Deviation ; S= significant association (P<0.05)

The result also demonstrated that the EGFR expression intensity in stomach tissue reached a peak at 10 days then decreased in the second period (20 days) in groups (T2, T3, and T4), whereas EGFR expression intensity in the group (T5) was still increasing during the time of treatment until 20 days as shown in **Figure (1).**

Similar results were observed in the duodenal ulcer that expression intensity increased at 10 days in groups (T2, T3, and T4) then decreased at 20 days. The intensity of expression in the group (T5) continued to increase with time of treatment as shown in **Figure (2).**

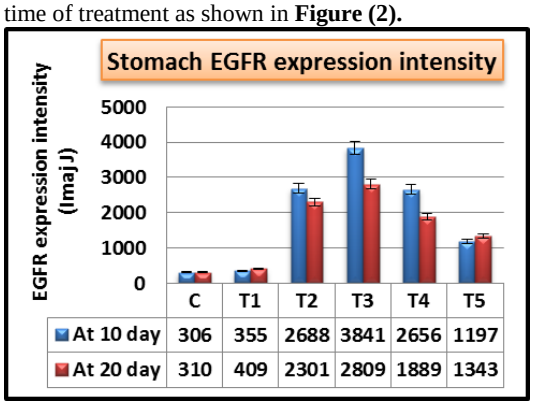

Figure 1. Effect mushroom extract and compounds on EGFR expression intensity of stomach ulcer after 10 days and 20 day.

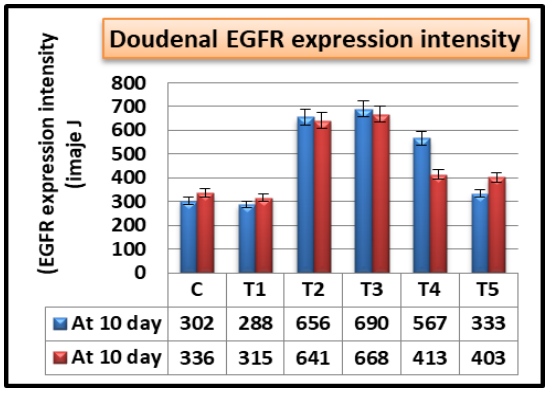

Figure 2. Effect mushroom extract and compounds on EGFR expression intensity of duodenal ulcer at 10 days and 20 day.

Anatomically, the study had been showing an increasing intensity of EGFR expression in the treated stomach and duodenal ulcer, and this increasing was related with clear mucosal healing especially in groups T3 at 10 and 20 days of the experiment (Figures 3,4)

The highest means of EGFR expression intensity in peptic ulcers recorded in T3 group may be due to the effect of the bioactive compounds of mushroom extract which induce the production of EGFR, and this may be related with the healing of ulcer and scar formation.

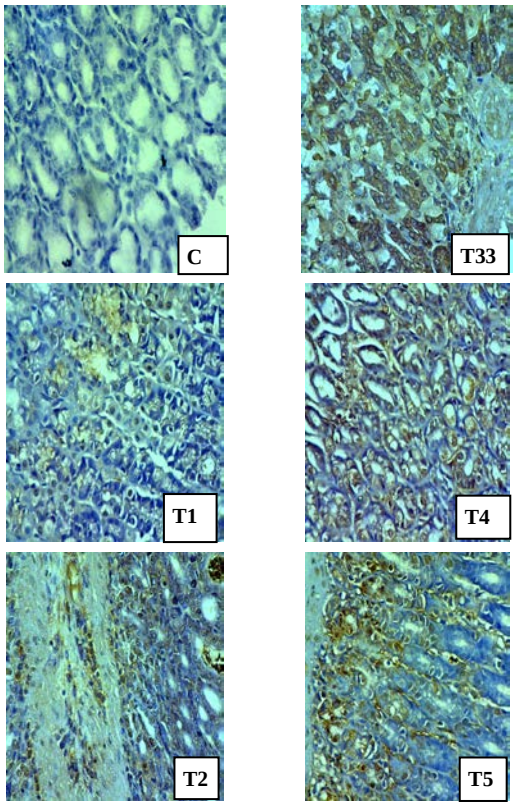

Figure 3. IHC staining for EGFR of stomach tissue at 10 days in treated groups (C: Control, T1: aspirin group,T2:omeprazole treated group,T3:*P.ostrateus* treated group,T4:*P.ostrateus* polysaccharide treated group and T5:chitin&chitosan *P.ostreateus* treated group) (X400).

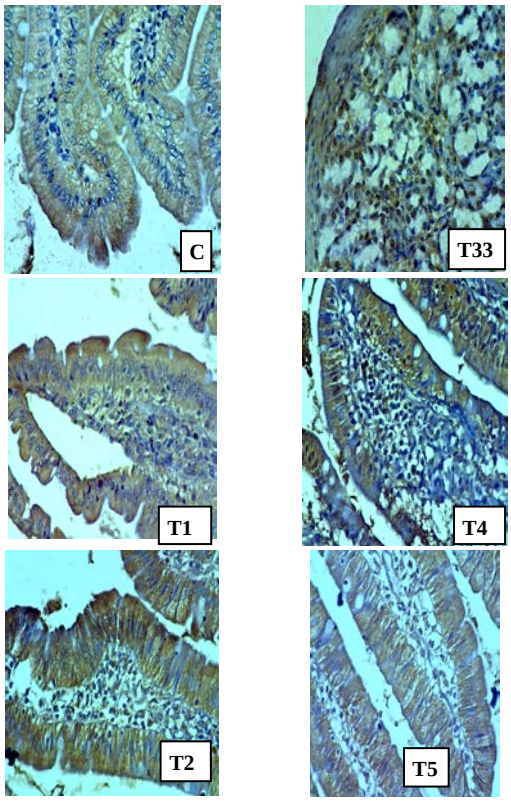

Figure 5. IHC staining for EGFR of duodenal tissue at 10 days in treated groups (C:Control,T1:aspirin group,T2:omeprazole treated group,T3:*P.ostrateus* treated group,T4:*P.ostrateus* polysaccharide treated group and T5:chitin&chitosan *P.ostreateus* treated group) (X400).

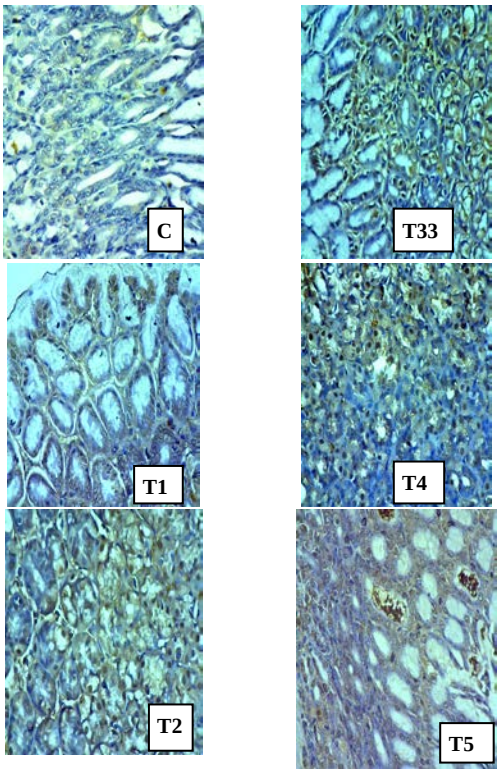

Figure 4. IHC staining for EGFR of stomach tissue at 20 days in treated groups (C: Control, T1:aspirin group,T2:omeprazole treated group,T3:*P.ostrateus* treated group,T4:*P.ostrateus* polysaccharide treated group and T5:chitin&chitosan *P.ostreateus* treated group) (X400).

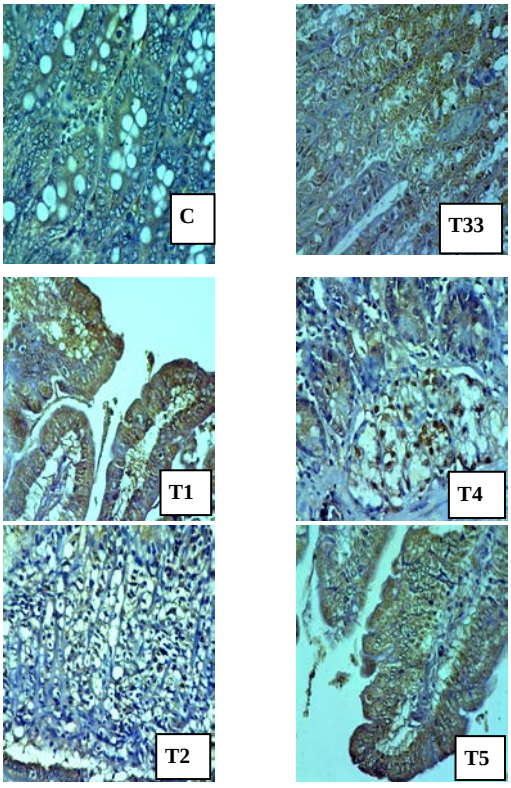

Figure 6. IHC staining for EGFR of duodenal tissue at 20 days in treated groups (C:Control,T1:aspirin group,T2:omeprazole treated group,T3:*P.ostrateus* treated group, T4:*P.ostrateus* polysaccharide treated group and T5:chitin&chitosan *P.ostreateus* treated group) (X400).

4. Discussion

Ulcer healing is an active and complex process that requires the coordinated interaction of various cellular and connective tissue components. During ulcer healing, processes including mucosal cell migration, proliferation and biochemical events are modulated by various growth factors, transcription factors and cytokines (Tarnawski,2005)

Many growth factors including Epidermal Growth Factor (EGF) contribute to the mechanism of ulcer healing as a result of the stimulatory effect on the process of mucosal repair, cell migration and proliferation as well as angiogenesis. The mechanism through which EGF initiate tissue healing is that when EGF binds to EGFR on the cell surface it will induce the activity of internal protein called tyrosine-kinase. This protein has triggered a signal transduction cascade that leads to a range of biochemical changes inside the cell, such as . intracellular calcium, glycolysis and protein biosynthesis and the expression of certain genes, including the epidermal growth factor receptor gene, which eventually leads to DNA synthesis and cell proliferation (Gupta *et al.*, 2019).

In this study, immunohistochemistry was used to assay the EGFR expression in aspirin induced gastroduodenal ulcer in male rat; the results showed a significantly reduced expression of EGFR in the aspirin group when compared with the treated groups. This may be due to suppression of endogenous NO synthesis that leads to delayed ulcer healing and high doses of aspirin impair normal NO activity, thereby suppressing the expression of growth factors (EGFR) in ulcer progression (Lanas *et al.*, 2000)

In normal control group (C), the expression of EGFR disappeared or appeared weakly only in proliferative zone and some of the parietal cells and this result are compatible with study of (Choi *et al.*, 2008) who found that EGFR was mainly localized in some mucous neck cells of the proliferation zone in normal gastric mucosa during immunohistochemistry examination.

The number of EGFR positive cells in the treatment groups significantly increased when compared with control and aspirin only groups throughout the experimental period. Research indicated that EGFR was closely related to the healing of impaired gastric mucosa and was of great importance in gastric mucosal protection (Alese *et al*.,2017)

Overexpression of EGFR has occurred during time of ulcer healing, and this is in agreement with the study of Choi, *et al*. (2014) who found significantly increased EGFR expression at 12 h, 24 h and 3 days after induction ulcer in the rat by using acetic acid. Szabo and Vincze,(2000) also recorded high expression of EGFR during early period of ulcer healing.

In this study, the expression of EGFR was increased in mushroom extract or compound treated groups; this might be suggestive of another probable mechanism by which the mushroom might have facilitated experimentally induced peptic ulcer healing. Moreover, the present study is the first to investigate the effect of extract of *P.ostrateus* on the expression of EGFR in peptic ulcer.

In the present study, the highest expression of EGFR in gastrointestinal ulcers was in group T3 and T6, treated by extract of *P.ostrateus*. Chemical composition of these fungi may be a good stimulator for the production of EGF, which can improve the quality of ulcer healing, and promote ulcer healing and tissue repair via binding with EGFR. Currently, EGFR is considered as an important evaluation indicator of the quality of ulcer healing (Faure and Lafont, 2013)

Lam *et al.* (2012) noticed that the extract of white button mushroom has an effective role in skin wound healing process which stimulates the proliferation of epidermal cells and suppresses cell death and inflammatory reaction. Study conducted by Hanawi *et al*.(2020) showed that the mushroom has curative effect on aspirin damaged tissue in stomach ulcer and preserved on normalcy histological architecture of stomach. The present results are comparable with Chen *et al.* (2016) who showed the healing of acute gastric lesions and chronic gastric ulcers is accompanied by an increased expression of EGFR in the ulcer area. Yan, *et al.* (2019) reported the correlation between the levels of epidermal growth factor EGF and the healing of the acute gastric mucosal injury in rats.

Fungal metabolites were known to produce a large and varied number of biologically active compounds that not only stimulate the immune system but also modulate specific cellular responses by interfering with particular signal transduction, antiulcer and a broad spectrum of healing activities such as asthmatic, anti-inflammatory, anti-obesity, anti-oxidative and anti-tumor (Yap *et al*., 2018; Samsudin and Abdullah, 2019)

This overexpression of EGFR occurred during the first period of experiment at 10 days, then decreased at 20 days; this decreasing was related to the proliferative role in complete tissue healing at 20 day, and this was consistent with study of Dharmani *et al*. (2013) who found that the mixture of eight probiotic bacteria including Lactobacilli, Bifidobacteria, and Streptococcus species heals acetic acid-induced gastric ulcer in rats and the gene expression of EGF and VEGF in 7 days more than in 14 day. This study was also compatible with the study of Zhang *et al*. (2012) who studied levels of EGF by immunohistochemistry in treated acetic acid gastric ulcer in rats at a different periods (1,2,4,6,10,14 and 23 days) and found a little increase on day 1 reaching a peak in 10 days. On days 14 and 23, the expression level and EGF peptide showed a little decrease.

5. Conclusion

Alcoholic extract of *P. ostrateus* has a significant effect on EGFR expression in ulcerated stomach and duodenum. Many studies on side effects, effective doses, mode of preparation on *P. ostreatus* should be done to improve it as an alternative treatment for peptic ulcers.

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