Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Comparative Study of Effects of Benzydamine HCl, Propolis Extract and Ginger Extract in Treatment of Induced Oral Ulcers of Albino Rats

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Abstract

Oral mucositis (OM) is a multi-etiological inflammatory condition. It is very painful and complicating patient's life, hence it was mandatory to find natural food supplements to manage this condition. Medicinal plants have been introduced in medical field as a potent anti-oxidant in co-treatment of this condition successfully. Ginger roots (*Zingiber officinalis* Roscoe) are one of the most anti-oxidant and anti-inflammatory plants with respect to its phytochemical constituents. This work aimed to the evaluation of histological and immuno-histochemical effect of natural product; Ginger oil extract in the healing of induced ulcers in Albino rat′s buccal mucosa. It also aimed to compare the obtained effect with that of marketed product; Benzydamine HCL spray (BBC, synthetic product) and Propolis sprays (Natural product). Forty eight adult male albino rats weighing 200-250 grams were divided into five groups. The Negative (-ve) and Positive (+ve) Control Groups: six rats in each, the first kept healthy and the second subjected to ulcer induction in the buccal mucosa. Three therapeutic groups 12 rats in each, Benzydamine hydrochloride Group (B) Gp: treated by BBC oral spray (3mg/animal/8 hours), Propolis (P) GP: treated by topical application of Propolis oral spray (3mg/animal/8 hours), Ginger Group (G) Gp: treated by topical application of Ginger oil spray (3mg/animal/8 hours). Each group was further divided into 2 subgroups A&B in which rats was sacrificed at 7 and 10 days following ulcer induction respectively. The buccal mucosa specimens were prepared and stained by H&E and immuno histochemical markers (Anti-PCNA) then examined by light microscope. The yield of the ginger oil resulting from the cold press method was 12% (v/w). Twenty four compounds were obtained after the GC/MS analysis with the identification of 20 compounds. 9,12-Octadecadienoic acid, oleic and palmitic acids TMS derivative (34.56%, 26.45% and 14.95%) were the major fatty acids, respectively. Histopathological examination of +ve control Gp revealed atrophy and complete necrosis of the epithelium overlying degenerated connective tissue (CT) with high infiltration of inflammatory cells and dilated BVs. These changes were markedly alleviated in therapeutic groups (B), (P) & (G) Gps. immunohistochemical results supported the histological results which showed significant statistically increased value of of PCNA expressions with relative increase in (G) Gp compared to (B) and (P) Gps at day 7 and 10. From the results, we can concluded that oral ulceration caused tissue degeneration as a result to the inflammatory responses leading to epithelial degeneration which was approved in +ve control Gp. Therapeutic application of BBC, Propolis and Ginger oil sprays markedly improved histological destruction revealed in +ve control Gp (confirming anti-inflammatory role of each of them). However, implement of natural herbs as Ginger oil resulted in maximum improvement compared to other therapeutic groups. Ginger oil can be used usefully as a topical drug for the healing of the oral mucositis.

KEY WORDS: Oral Mucositis, Benzydamine HCL, Propolis, Ginger

1. Introduction

Oral Mucositis (OM) is a painful open sore on the buccal mucosa of the oral cavity prompted by an inflammatory mucosal disruption triggered by a variety of factors. It may cause a negative reflection on patients' quality of life in severe cases [1]. Furthermore, OM can occur as a result of mucosal infection or systemic involvement as a result of compromised immunity [2]. It is a relatively common finding in dental practice with a reported prevalence of 25% to 80%, occurring almost in one of five people in the population[3].

There are several factors responsible for aphthous ulcers, most of them are traumatic injury, chemical, electrical, thermal, genetic predisposition,

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Receive Date: 09 May 2023, Revise Date: 13 June 2023, Accept Date: 18 June 2023

DOI: 10.21608/EJCHEM.2023.209140.7940

food allergies, microbial factors, anxiety and vitamin B12 deficiencies[4].

There are many treatment modalities are practiced in the management of OM. Two lines therapy options are applied; topically and systemic agent. The first line; topical agents, is cheap, effective and safe but they are easily inevitably rubbed or rinsed away[5]. It consists of antiseptics and anti-inflammatory analgesic drugs. The second line includes systemic immunomodulator, systemic antibiotic and corticosteroids[6].

Systemic treatment options have a lot of adverse effects such drug-drug interactions, drug allergy and effect on the health of patients especially with chronic diseases[7]. Most of herbal medicines have advantages of being safe, effective and rapid actions [8]. This study involved the comparison between topical chemical drug Benzydamine hydrochloride (BBC) and natural products (Propolis and Ginger oil), evaluation of the effectiveness of ginger essential oil and correlate with its phytochemical constituent.

BBC is a topical anti-inflammatory oral spray drug has analgesic and antiseptic effect. The negligible side-effects was the cause of being widely used [9]. According to the precaution from the Amoun Pharmaceutical Company, BBC should not be prescribed in the children below 6 years or elderly patients as it may affect swallowing and also for pregnant and lactating patients.

Propolis is a naturally bee product. It is a hard resinous substance consisting of wax and plant extracts[10]. It was traditionally used as an antibacterial**,** antioxidant, anti-septic, anti-inflammatory, anti-mycotic and antimicrobial agent [11,12]. The previous studies proved these biological activities of propolis [13,14]. Propolis extract possess antimicrobial activity against gram-positive cocci; *Streptococcus mutans* ; facultative anaerobic bacterium commonly found in the human oral cavity and a significant related to tooth decay[15]. It also used as an alternative measure in the prevention of dental caries. It gave promising results as an intracanal medicament[16]. In several small case studies and pilot clinical studies, the efficient effect of propolis was clear in the treatment of gingivitis and oral ulcers [17].

Ginger (*Zingiber officinalis* Roscoe, family Zingiberaceae) rhizome is one of the most famous species [18]. It is related to south-eastern Asia, China and India[19]. It is traditionally used as dietary supplement[20], a condiment for various foods and beverages and in the treatment of hypertension, arthritis and gastrointestinal disorders [20,21]. It also has anti-inflammatory and antimicrobial activities[22]. Common medicinal forms of ginger include fresh root, dried roots and powders. Intact rhizomes are the best source of the essential oil of ginger because of the existence of substantial amounts of the oil in the peel[18]. Ginger oil extract was prepared from fresh rhizome by different methods; steam distillation, cold pressing, microwave hydro-distillation and by solvent extraction[23]. Green technologies help in the increased of the demand on natural products alongside the worldwide market. Cold pressing is a simple technique which gives the advantage of providing massive amounts of bioactive compounds without using thermal or chemical treatments [24–26].

Fresh ginger rhizomes constituents were divided into two groups; volatiles and non-volatiles. The first group includes monoterpenoid hydrocarbons and sesquiterpene which gave the specific aroma and taste. Gingerols, shogaols, paradols, and zingerone were pungent compounds that represent the non-volatile group [27]. These phytochemical constituents provide ginger anti-inflammatory, antimicrobial and antioxidant activities[28,29]. The high antioxidant and antimicrobial activities were proved in the ethanol (10%) extract and in the juice prepared by cold press technique[30,31]

The antimicrobial effectiveness of ginger have also been evaluated and gave great results against gram negative pathogens routinely encountered in periodontal infections such as *Porphyromonas endodontalis Prevotella intermedia*, and *Porphyromonas gingivalis*, [15].

Little literature was available regarding the cold pressed technique for ginger oil; constituents and pharmacological activities. El Makawy et al. and Ramadan [20,26] were only discussed the constituent of the ginger oil using this technique and reported its effect as antiepileptic drug on testicular gene expression, and sex hormones and in mice

Thus, the aim of this study was to evaluate the ameliorating effects of ginger oil comparable to other commercial therapeutic topical sprays; BBC and propolis, on oral mucositis in rat models and determine its phytochemical constituents.

MATERIAL AND METHODS

Materials

Formicreseol: as a chemical caustic agent. It was consisting of mixture of formalin, cresol and glycerine that causing discontinuity the overlaying buccal mucosa[32].

BBC® spray (Benzamide HCL); marketed synthetic drug, was purchased from El-Ezaby pharmacy. Intraoral topical spraying of BBC® was applied (3mg/1puff/animal/8 hours) [33].

Propolis extract; an international marketed product, was purchased from Comvita USA online website. Intraoral topical spraying of Propolis® was applied in a dose of (3mg/1puff /animal/8hours)[34].

Fresh rhizomes were purchased from local herbalist market (2019) and authenticated by the Agriculture Research Center, Egypt. Ginger extract (oil) has

been prepared at Pharmacognosy Department, National Research Center. It was applied as Intraoral spray in a dose of (3mg/animal/8 hours) [35].

Animals

Fourty eight adult male Albino rats (200- 250 gm) were supplied by the animal house at the National Research Centre (Cairo, Egypt). They were located in separate cages (n=8/ cage) under hygienic conditions; temp. (22–25°C), humidity (50±5%), with a 12 h light/dark cycle and fed a standard laboratory diet and tap water ad libitum. This study was established and admitted by the "Research Ethics Committee", Faculty of Dentistry, Ain Shams University (Approval number: FDASU – Rec ID 091809).

Methods

Ginger oil Extraction

Fresh rhizomes (500g) were subjected to cold press through large machines at low temperature [36]. High-quality oils were obtained and stored at -20ºC [37]. The oil (0.2 μL) was analyzed by gas chromatography /mass spectrometry (GC/MS) with methyl silicone column [38].

Identification of the components of ginger oil.

The main components of the essential oil were identified by GC/MS with methyl silicone column at the central laboratory, National Research Centre with the following specifications. Instrument: Gas Chromatography coupled with a Mass Spectrometers; Finnigan mat SSQ 7000, Digital DEC EL eV 70 for GC/MS analysis of volatiles, carrier gas; Nitrogen (1 mL/min). Injector Temperature 220 °C; Volume injected was 1µl. Ionization Energy, 70eV. Oven temperature was programmed at 60°C isothermal for 3 min. then heating to 260°C at a rate of 4°C /min. then isothermal at 260°C for 5 min.

Identification of the obtained constituents was based on the comparison of their retention times, and mass spectral fragmentation patterns with those of the available database library (Wiley Int. USA) and NIST (Nat. Inst. St. Technol., USA)], and with those reported, and/or those described by Adam[39,40]**.**

Experimental Design

After one week; acclimatization period, rats were divided into three groups (12 rats /each), besides negative and positive control groups (6 rats/ each). The negative control (-ve control); were kept in a hygienic conditions for 10 consecutive days.

Group 1-4: Rats were anesthetized with sodium thiopental and ulcers were induced in the left buccal mucosa by topical application of a number 4 pellet soaked in full strength Formocresol 3 times daily for 3 days. Ibuprofen at a dose of 15mg/kg (235 ml in 500ml water) was used as a pain killer [41]. Group 1 (Positive Control; +ve control): six rats were exposed to Formocresol ulcer induction. Group 2 (Benzydamine hydrochloride, Gp B): twelve rats were subjected to oral ulceration. Rat's buccal mucosa of this group treated by BBC® oral spray 30mg/kg three applications daily[33]. Group 3 (Propolis; P): twelve rats were subjected to oral ulceration. Rat's buccal mucosa treated by Propolis® oral spray 30mg/kg three applications daily[34]. Group 4 (Ginger; G): twelve rats were subjected to oral ulceration. Rat's buccal mucosa treated by Ginger oil spray 30mg/kg three applications daily [35].

Each group was subdivided into 2 subgroups, 6 rats/ each (A1 to A4) and (B1 to B4) according to the time of scarification (day 7 and day 10, respectively).

Sample preparation for staining by Hematoxylin & Eosin and Anti-PCNA

At the end of the experiment; day 7 and day 10, rats were sacrificed by a high dose of anesthesia. The collected tissue samples of all rats were eradicated and fixed in buffered formaldehyde solution (10%) for 48 hours. Tissue samples were then appropriately washed by running water then dehydrated by rising concentrations of alcohol. Alchols was cleared by xylene then the specimens impregnated and embedded in the paraffin wax blocks[42]. Sections (4 µm thick) were obtained and put in xylene to reduce concentrations of alcohol(100% to 70%) then washed with distilled water to eliminate the paraffin wax[43]. Finally, specimens were stained according to the published procedure by Hematoxylin & Eosin (H&E) [43] and immuno histochemical markers for cell regeneration (Anti-PCNA) [44].

Statistical analysis

The Mean value of both vacuolated epithelial cells and Anti- proliferating Cell Nuclear Antigen (Anti-PCNA) positive cells were examined for normality by observing the distribution of data and utilizing tests of normality. The comparison between five groups was done by One-way ANOVA test. The pair-wise comparison between two significant groups was applied by using Bonferroni's post-hoc test. The significance level was set at $P \leq 0.05$. IBM® SPSS® Statistics Version 21 for Windows was used to conduct the statistical analysis.

RESULTS AND DISCUSSION

The extract percentage obtained from fresh rhizome of *Zingber officinalis* was 12% (v/w). The analyzed oil by GC/MS with methyl silicone column revealed the presence of 24 components. Twenty compounds were identified representing (98.25%) of the total oil composition as shown **(Table 1)**.

The oil composed mainly of sesquiterpenes oxygenated derivatives and fatty acids. Linoleic; 9,12-Octadecadienoic acid, (34.56%), oleic (26.45%) and palmitic (14.95%) acids TMS

derivative were the major fatty acids. These 3 compounds represent 75.96% of all identified compounds. The remaining compounds represent low percentage. These results of the fresh ginger roots extracted by cold press method were resemble to that analyzed of the commercial essential oil purchased from EL-Captain Company [20] and with published data for the analyses of fresh ginger root of Faisalabad, Pakistane extracted by hydrdistillation method [45] and of Egypt extracted by soxhlet apparatus [46] with some difference in the percentage of constituent. The variability in these percentages is affected by the nature of roots being fresh or in dried form and the climate difference[46].

Table 1: The identified compounds resulting from GC/MS analysis of the fresh *Zingiber officinale* (ginger) extract by cold press method

R_t	Compound	Area %	Formula
8.811	Butanoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	1.51	$C_{10}H_{24}O_3Si_2$
9.361	Camphene	0.74	$C_{10}H_{16}$
11.132	*β*-mycrene	0.35	$C_{10}H_{16}$
12.021	α-phellandrene	3.91	$C_{10}H_{16}$
13.151	α-zingiberene	1.18	$C_{15}H_{24}$
15.592	cis-.beta.-Farnesene	0.62	$C_{15}H_{24}$
16.271	Geraniol, TMS derivative	1.17	$C_{13}H_{26}OSi$
16.444	Geranyl isobutyrate	1.25	$C_{14}H_{24}O_2$
16.617	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)-	0.38	$C_{15}H_{24}$
16.813	trans-Caryophyllene	1	$C_{15}H_{24}$
17.062	Nerolidol	0.93	$C_{15}H_{26}O$
23.708	Palmitic Acid, TMS derivative	**14.95**	$C_{19}H_{40}O_2Si$
25.615	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	**34.56**	$C_{21}H_{40}O_2Si$
25.667	Oleic Acid, (Z)-, TMS derivative	**26.45**	$C_{21}H_{42}O_2Si$
25.848	Octadecanoic acid, trimethylsilyl ester	3.36	$C_21H_{44}O_2Si$
28.259	1,2-15,16-Diepoxyhexadecane	1.03	$C_{16}H_{30}O_2$
30.799	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]m	2.22	$C_{27}H_{52}O_4Si_2$
32.894	6,9,12-Octadecatrienoic acid, phenylmethyl ester, (Z,Z,Z)-	0.44	$C_{25}H_{36}O_2$
36.247	Cholest-5-en-3-ol (3.beta.)-, 9-octadecenoate, (Z)-	0.42	$C_{45}H_{78}O_2$
37.091	Methyl 10,12-pentacosadiynoate	1.78	$C_{26}H_{44}O_2$

Identified = 98.25% **Unknown= 1.75%**

Histopathological results

Hematoxylin & Eosin (H&E)

Light microscopic examination of the buccal mucosa sections stained with **H&E (X 200)** of all groups under investigation were done. In the negative control group **(Fig.1a)**, epithelium of buccal mucosa is normally covered by corrugated continuous keratin layer (black arrow) were observed. Intraepithelial vacuolation were rarely encountered. Normal histological structure was observed in the underlying connective tissue (CT) and attached to the underlying muscle fibers by a loose connective tissue component. The epithelium and the lamina propria were separated with broad numerous rete pegs. Fibroblasts were regularly arranged with basophilic stained cytoplasm, well- organized collagen fiber were detected.

In the positive control group (+ve control), the examination of histological stained sections of the sub group A at day 7 revealed that most of the specimens showed discontinues epithelial which is covered by fibrinopurulent membrane covering inflamed C.T which was highly infiltrated with mixed inflammatory cells mainly neutrophils. The adjacent epithelium showed reactive atypical findings such as basal cell hyperplasia, nuclear hyperchromasia, and pleomorphism. Heavy infiltration by inflammatory cells was detected in the underlying C.T with marked vasodilation in the blood vessels. The lamina propria showed dilated blood vessels with interrupted endothelial lining and extravasated RBCs **(Fig. 1b)**. On the other hand, some specimens showed small ulcer filled with granulation tissue (black arrow) highly infiltrated with inflammatory cells and discontinuity of epithelial layer (arrowheads).. The epithelium of these specimens showed degeneration in some areas while granulation tissue, inflammatory cells and dilated blood vessels with interrupted endothelial

lining appeared in lamina propria of the mucosa **(Fig. 1c)**.

Examination of the sub group B sections at day 10 showed healing ulcers or the edge of ulcers demonstrate sub-epithelial fibrin deposition, granulation tissue with intraepithelial hemorrhage and reactive cell atypical findings, complete re-epithelization, formation of epithelial ridges and granulation tissue while the lamina propria was fibrous, moderate infiltration of inflammatory cells and extravasated RBC's **(Fig.2b)**. In other specimens, the epithelium showed increases in thickness with hyperkeratosis and the muscle layer appeared separated from the submucosa. The epithelium showed intact basal cell layer and mitotic activity while the connective tissue appeared fibrous with dilated blood vessels, extravasated RBCs and heavily infiltration with inflammatory cells **(Fig. 2c).**

In the BBC **group (Gp B),** examination of sections at day 7 stained (sub-group A) showed complete mucosa ulceration (black arrows); the epithelium appeared necrotic overlying C.T with marked vasodilation bl. vs (arrowhead) that was congested with coagulated blood. The epithelium showed irregular basement membrane with short and wide rete pegs with different thickness. In some areas, basilar hyperplasia was detected. Faintly stained cytoplasm in granular cell layer with ill-defined keratohyaline granules and keratinized cell layer showed hyper keratosis. The Fibrous connective tissue showed vasodilation with highly infiltration of inflammatory cells with marked decrease when compared to control +ve group **(Fig. 1d).**

Examination of the subgroup B at day 10 revealed several variations with the same percentage; most of the specimens showed areas of epithelial discontinuity which is covered by fibrinopurulent membrane (C.T) **(Fig. 2d)**. Specimens showed separation of epithelial layers from the underlying Lamina propria filled with either granulation tissue or necrotic tissue, hyperemic C.T with interrupted endothelial lining and heavy infiltration with inflammatory cells. The epithelium showed disorganization of basal cell layer and some areas of degeneration. Granular cell layer appeared with barely stained cytoplasm and poorly distinguished keratohyaline granules**.** In some specimens, complete ulceration was found in epithelium and (C.T), Necrosis and detachment of epithelium and underlying granulation tissue with heavily infiltration with inflammatory cells **(Fig. 2e).**

Histological sections of subgroup A of the Propolis group (GP P) showed elevation in the thickness of epithelium compared with its correspondent BBC gp. Broad, irregular epithelial ridges were appeared in some areas while in other areas appeared tall and narrow **(Fig.1e).** Intraepithelial edema with significant hyperplasia in the basal cell layer was observed. Few mitotic figures were detected in few cells within the para basal cell layer**.** Granular cell layer showed keratohyaline granules which stained basophilically**.** Thin keratotic layer with some areas of separation was showed overlying the stratified epithelium. The connective tissue showed few capillaries and blood vessels with less organized collagen fibers. Degeneration of some areas was also detected. Collagen fiber bundles were oriented transversely with some areas of separations in sub mucosa. Examination of the subgroup B at day 10 revealed that most of the specimens showed that epithelial layer was thick, continuous, elongated or broad rete pegs with marked mitotic activity in basal & parabasal layers **(Fig. 2e).** The C.T was fibrous, showed increase in vasculature, extravasated RBCs and infiltration with inflammatory cells.

The histological sections of the subgroup A of the Ginger group (G) **(Fig. 2g)** showed hyperkeratotic and corrugated epithelium with marked increasing in the epithelium thickness (black arrows) when compared with its corresponding subgroups. Broad or elongated epithelial rete bigs appeared being irregular or flat in different areas **(Fig. 1f).** The basilar hyperplasia was detected in the basal cell layer with marked increased mitotic divisions within the para basal cell layer. Perinuclear haloing in few cells in basal cell layer were detected. Granular cell layer appeared faintly stained with few basophilically stained keratohyaline granules. The cornified cell layer appeared compact and thick. The connective tissue appeared mildly hypermic with vasodilated blood vessels were detected. While the examination of subgroup B at day 10 revealed that most of the specimens showed that epithelium was hyperkeratotic. The keratin layer appeared non-uniform with separations from epithelial thickness, thick and continuous. The basal layer showed hyperplasia with mitotic activity. The lamina propria appeared fibrous and more or less organized. Blood vessels appeared slightly dilated and filled with RBCs. Few separations were found in between muscle fibers

Immunohistochemical staining results (anti PCNA)

The proliferating cells were detected by using anti-PCNA immune-stain. The brown nuclear staining revealed the positive reactions.

LM examination of the -ve Control specimens **(Fig. 3a).**showed that distinct positive brown reaction in the basal and parabasal cell layers was observed, while prickle and granular cell layer showed negative to slightly positive PCNA reaction in both epithelium and lamina propria**.** Some cells with positive reaction within the CT.

The subgroup A at day 7 of the +ve control specimens showed a fewer positive reaction in the epithelium of ulcer area compared with the

surrounding epithelium while the connective tissue showed a strong positive reaction beneath the ulcer while negative reaction was present in granular cell layer **(Fig. 3b).** In the non-ulcerated specimens, the epithelium showed numerous positive reactions in basal and parabasal cell layer while fewer positive cells were seen in the prickle cell layer. The subgroup B at day 10 revealed that, in the ulcerated specimens, the epithelium of ulcer area showed a fewer positive reaction compared with the surrounding epithelium while the connective tissue showed a numerous positive reactions of PCNA antibody In some areas of basal cell layer while prickle and granular cell layer showed negative reaction. Also few positive reactions appeared in the (C.T) (**Fig. 4a).**

Examination of PCNA immunolocalization sections of subgroup A of the Gp (B), BBC, revealed that in the ulcerated specimens, there was complete negative reaction to PCNA immunolocalization and positive reaction in the surrounding epithelium. The ulcer edge showed positive reaction in basal and prickle cell layers, negative reaction in granular cells and few positive reactions in C.T **(Fig. 3c).** Examination of subgroup B at day 10 in this group revealed that, the ulcerated specimens showed almost negative reactions in the epithelium except few areas of positive reactions in basal cell layer and few cells in the C.T. On the surrounding epithelium there were moderate expression of PCNA antibodies in basal and suprabasal cell layers, negative reaction in prickle and granular cell layer and little reaction in the C.T. **(Fig. 4b).**

While, the ulcerated specimens of the subgroup A of Propolis Group at day 7 showed complete negative reaction at the ulcer site and positive reaction at the epithelium and C.T. The ulcer edge showed numerous positive reaction in basal and prickle cell layers while negative reaction were seen in granular cells and some of the prickle. The connective tissue also showed numerous positive reactions **(Fig. 3d).** Examination of subgroup B at day 10 in this group revealed that, some ulcerated specimens showed numerous positive reactions at granulation tissue filling the ulcer, the epithelial ulcer edge and C.T. The epithelium showed numerous positive reactions in basal and prickle cells and negative reaction in granular cells and superficial cells of prickle layer. Connective tissue also showed numerous positive reactions **(Fig. 4c).**

The examination of the subgroup A sections in the Ginger extract Group showed complete negative reaction at the ulcer area of the ulcerated specimens; the blood vessels engorged with coagulated blood in the C.T., while positive reaction appeared at the epithelium the ulcer edge. The epithelium of ulcer edge showed numerous positive reaction in basal and prickle cell layers while negative reaction were seen in granular cells and some of the prickle. The non-ulcerated specimens in this subgroup showed numerous positive reactions in the basal and suprabsal cells (except for small areas) of the epithelium, negative reactions in prickle cells. Many positive reactions appeared in C.T **(Fig. 3e).** The examination of subgroup B at day 10 showed that most of the ulcerated specimens were non ulcerated and showed numerous positive reactions in the basal and suprabasal cells while negative reaction appeared in prickle cells. C.T. Cells showed few positive reactions **(Fig. 4d).**

The data of the statistical analysis were calculated and summarized in Tables (2-3). **Table (2)** showed that mean values of PCNA immunolocalizalion was in the descending order of the all subgroups. While **Table (3)** showed the mean values of PCNA expression of experimental groups (gr. 2B, 3B) showed a high significant decrease than that of the control +ve group (gr. 1B). The numerical data were also designed in Bar chart **(Fig.5)**

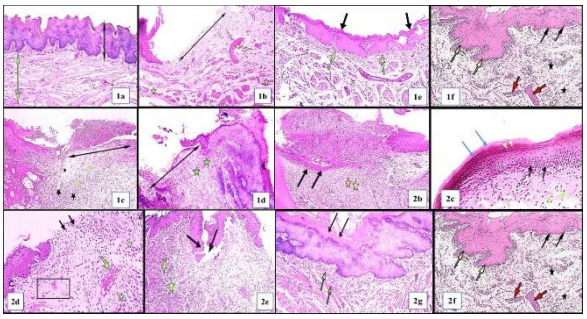

Fig. (1-2): Photomicrographs of rat's buccal mucosa stained with H&E, x 400. **(1a**) negative control group, **(1b)** +ve control Gp at 7 days, showed interrupted epithelium and keratin, demarcating remnants of the ulcerated region (black arrow) and the epitheliod cells extending deep into lamina propria (yellow arrow). **(1c);** the same Gp, there was an ulcer filled with granulation tissue (black arrow). Lamina propria highly infiltrated with inflammatory cells (arrowheads). **(1d)** Gp (B) at day 7 showed complete ulceration of mucosa with marked vasodilation of B.Vs and extracellular edema in CT. **(1e)** Propolis Gp at 7 day was same as -ve control Gp with few intracytoplasmic vacuolations in the epithelium (arrow). **(1f)** Ginger gp at 7 days showed epithelium and CT image similar to -ve control Gp. **(2b)** +ve control Gp at 10 days showed formation of epithelial ridges and granulation tissue (black arrows) and heavy infiltration with inflammatory cells (stars), also in **(2c)** of the same Gp showed: intact basal cell layer with increasing in thickness with hyperkeratosis (black arrows). The muscle layer appeared separated from the submucosa (stars). BBC (B) Gp at 10 day **(2d)** showed epithelium detachment with Lamina

propria filled with granulation tissue (black arrow), dilated bl.v (arrowheads) and inflammatory cells (stars), also in **(2e)** of the same Gp showed: separation of epithelial cell layers from Lamina propria (black arrow) which is heavy infiltrated with inflammatory cells (star); Propolis Gp at 10 day **(2f)** showed continuous, long and irregular epithelial ridges (black arrows). Increase vasculature in C.T with heavy infiltration of inflammatory cells (arrowheads); Ginger Gp at 10 day **(2g)** showed: hyperkeratotic and corrugated epithelium with thick and continuous layers (black arrows). Lamina propria is fibrous and mildly hypermic with (green arrows)

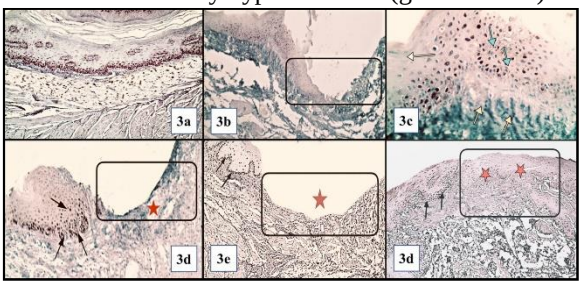

Fig. (3): Photomicrographs of rat's buccal mucosa stained with Anti- PICNA (x 200). **(3a)** at day 7, -ve control Gp showing positive reaction in all basal and few para- basal cells of the epithelium and some cells with positive reaction within the CT. **(3b)** +ve control Gp in, showing some positive reactions (black arrow), alternating with negative ones (red arrow) in basal cell layer. Minimal or rare positive reaction was detected in CT. (**3c**) B Gp showing positive reaction (black arrow), in basal and parabasal layers of epithelium as well as CT. **(3d)** P Gp, positive reaction (arrows) basal and parabasal layers of epithelium were shown. CT also displayed many positive cells**. (3 e)** G Gp, strong positive reaction (arrows), in basal and parabasal layers of epithelium was observed. CT also displayed many positive cells..

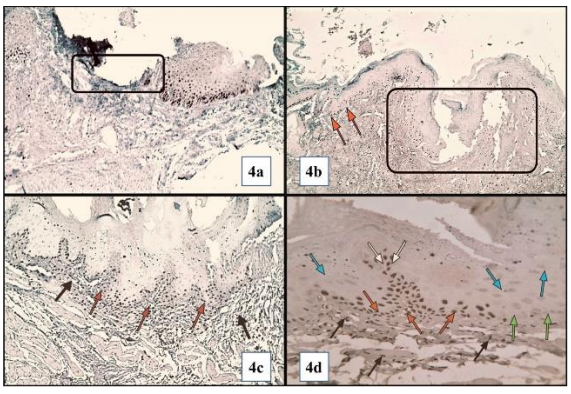

Fig. (4): Photomicrographs of rat's buccal mucosa at day 10 stained with Anti- PICNA (x 400). **(4a)** +ve control Gp showing positive reaction in all basal and few parabasal cells of the epithelium, and some cells with positive reaction within the CT. **(4b)** B Gp is showing some positive reactions (red arrows), alternating with negative ones (red arrow) in basal cell layer. Minimal or rare positive reaction was detected in CT. **(4c)** P Gp is showing positive reaction in basal and parabasal layers of epithelium (red arrows) as well as CT (black arrows). **(4d)** G Gp is showing strong positive reaction (colored arrows), in basal and parabasal layers of epithelium. CT also displayed many positive cells (black arrows).

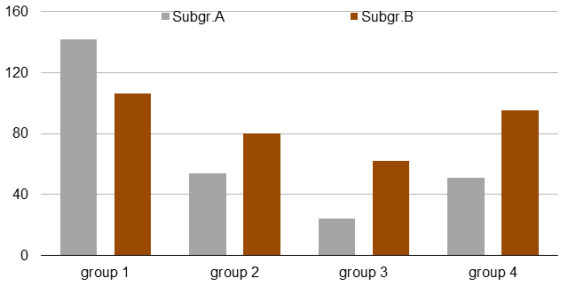

Fig. (5): Bar chart showing means of PCNA expressions in each group

Discussion

This study focused on the buccal mucosa because it's one of the most common areas in cases of OM affected by accidental, iatrogenic and chemical trauma. It also considered as a mirror of the general body health. It is an extremely painful condition[47,48]**.**

Oral ulcers is managed mainly by topical application of benzydamine hydrochloride gels, sprays and pastes were advocated [49]. Nutritional supplements as Propolis extract sprays in the form of multivitamins that would improve the healing were also prescribed for ten days is known to possess a good prognosis[15].

Recently natural herbs have been introduced in medical field as a co-treatment of many conditions and potent anti-oxidant [50]. Our study aims to compare the curative effects of Benzydamine hyrocholoride spray, propolis spray and natural drug, ginger extract on rat's buccal mucosa with formocresol-induced ulcerations. Ginger was reported that have immunostimulants, cell proliferators, anti-inflammatory and anti-microbial agents, highly antioxidant activity and anti-pyretic actions [51].

The reported results of phytochemical analyses revealed that ginger oil contains high amounts of sesquiterpene hydrocarbons and relatively low monoterpene hydrocarbons. The differences in the chemical constituent of oil extracted related to the nature fresh or dry ginger[26].

Table (2): Comparison of PCNA immunolocalization between the experimental groups and control group at day 7 (sub group A).

	Paired Differences					T	df	Sig. (2-tailed)
	Mean	Std. Interval of Deviation	Std. Error Mean	95% Confidence the Difference				
				Lower	Upper			
Pair 1 1A-2A	87.57143	36.32656	13.73015	53.97496	121.16789	6.378	6	0.001
Pair 2 1A - 3A	117.42857	23.21535	8.77458	95.95796	138.89919	13.383	6	0.000
Pair 3 1A - 4A	90.85714	32.26675	12.19568	61.01538	120.69891	7.450	6	0.000

.

Table (3): Paired comparisons of PCNA immunolocalization mean values of sub groups (2B, 3B and 4B).

	Paired Differences					T	df	Sig. (2-tailed)
	Mean	Std. Interval of Deviation	Std. Error Mean	95% Confidence the Difference				
				Lower	Upper			
Pair 1 1A-2A	18.42857	18.13704	6.85516	1.65460	35.20254	2.688	6	0.036
Pair 2 1A - 3A	-14.57143-	19.60321	7.40932	-32.70137	3.55851	-1.967	6	.096
3Pair A4 - A1	-33.00000-	26.70830	10.09479	-57.70106	-8.29894-	-3.269	6	.017

Fresh ginger oil contained oxygenated compounds (29%) while dry ginger oil (14%). α-zingiberene, aα-curcumene, geranial, and camphenes were reported to be the main compounds in different studies using soxhlet method or blinding with ethanol [38]. The obtained data of our study was in agreement with that published by El Makawy et al.[20]. They were found that Linoleic and oleic acids were the main fatty acids (81%), palmitic and stearic acids (14%) were the main saturated fatty acids. It was found that ginger oil contains high amount of polyunsaturated and monounsaturated fatty acids (43.1%, 41.0%), respectively.

Pathological changes at +ve control Gp, including keratinized thin layer[52], delayed healing, necrotic areas in the epithelium, C.T infiltration and dilated bl.v. at day 7 was in an agreement with **Duarte et al** [53]**.** They made a stomaties model in Wistar rats and he observed extensive ulceration covered by necrotized, fibrous tissue whose connective tissue was showing interstitial edema. Marked congestion was observed in blood vessels with heavy infiltration by inflammatory cells. On day 10, the specimens of the present study (Untreated group) displayed complete re-epithelization, formation of epithelial ridges and granulation tissue, irregular distrophic keratotic layer was observed overlying the ulcerated areas.

De Carvalho et al.[54] also examined the effect of formocresol on the mucosa of rats. He reported ulcerated wounds covered by granulation tissue. Intense and mixed inflammation in the underlying C.T. with marked dilated blood vessels and deposition of less organized collagen fibers at day 3. On day 5, the ulcers were covered with thick keratin layer. Next, the epithelial coverage showed acanthosis or degeneration in some areas. The connective tissues showed new proliferated congested bl.v. with heavy chronic inflammation. On day 11, ulcers covered by an irregular keratinized stratified squamous epithelium. The dermis displayed collagen proliferation which was mature and abundant (which equivalent to day 10 in the present study).

Benzydamine group (B gp) showed slight improvement than group 1 at day 7, the specimens showed signs of regeneration in the ulcerated area; re-epithelization was detected in the basal cell layer. Heavy rete pegs were shown at the ulcer edges. The dermis appeared loose with inflammatory cell infiltration and newly formed blood vessels. On day 10, marked improvement in wounds healing represented by ulcer closure in a large proportion of specimens, increased vasculature (angiogenesis) and decreased infiltration of inflammatory cells in the C.T compared with group 1 these findings were supported by **Liu et al.,**[55] **and Yaprak et al.,**[56] who examined the effect of benzydamine hydrochloride gel on ulcerated mucosa. Complete healing was detected on day 12 with a marked decrease in the inflammatory responses. New capillaries were formed at the surface with re-epithelization. The control group failed to show complete healing of the mucosa (this means that ulcers in this study recovered completely 2 days faster than previous studies).

Propolis group (P Gp) in the present study showed better and faster healing compared to control +ve and slight improvement than B Gp especially at day 7. Re-epitheliation was detected in the ulcerated areas at basal and parabasal cell layers. The underlying dermis appeared loose in some areas and dense in others with few inflammatory cells and extravasated red blood cells. Araujo et al., [57] studied the effect of Propolis as anti-inflammatory in different models of acute and chronic inflammation; formocresol-induced arthritis and paw edema induced by PGE2 or radiation. He found that propolis having the same effect as the positive control in these experiments.On day 10, massive development revealed in the epithelium and C.T, which was appeared as significantly increase in epithelial cell proliferation and reduction in the mean value of vacuolated epithelial cells. Apparent increase in the mature collagen fibers relative to group 1 was clearly detected. Awaad [42] found that Propolis helped in acceleration of healing of induced oral mucositis through epithelization in the ulcerated areas in the form of basal and prickle cell layers with continuous keratin layer of even thickness. The dermis showed similarities to the -ve control group, with reduced in infiltration of inflammatory cells. The endothelial lining of blood vessels is well developed. Collagen fibers were well formed in the CT of this study.

Ginger group (G Gp) in the current study revealed that therapeutic application of ginger accelerates healing of buccal mucosa at day 7. Re-epithelization with heavy rete pegs was observed. Separated keratin layer with deeply stained keratohyaline granules in granular cell layer was observed with hyperkeratosis in some areas. The dermis appeared loose with new vascolarilization and mild infiltration with inflammatory cells. Shredah and El Deeb [58] approved that ginger has marked alleviated effects in healing of oral mucositis caused in Methotrexate treated albino rats. Histopathological examination revealed markedly attenuated more or less normal appearance when compared to Methotrexate treated rats. The epithelium regeneration reached the normal appearance which showing intact and thick keratinous layers at day 5. However, minimal cytoplasmic vacuolation and signs of nuclear pyknosis was also shown. Deeply stained basophilic keratohyaline granules were detected in the the granular cell layer at day 11.

By comparing the immunohistochemical results of the current study; it was found that there was a high significant decrease of PCNA expression in Propolis group at day 7 compared to Benzydamine treated group. This is matched with Awaad[42] who found that propolis treated rats revealed fewer PCNA positive cells in the basal and parabasal layers of the epithelium as compared to the -ve control , which was also statistically confirmed. This approved the deduction in proliferation potential of the epithelium and accompanied by the accumulation of inflammatory end-products. The actively-dividing basal and parabasal epithelial cells, rendering them incapable to perform their function properly [44].

On day 10 of our study, propolis group revealed a significant decrease of PCNA expression than that of Benzydamine treated group.

The present study revealed that, therapeutic ginger group accelerates the healing of buccal mucosa at day 7 than what was observed in BBC and Propolis treated groups. The immunohistochemical results showed significant increased value of PCNA expressions compared to Benzydamine and Propolis treated groups at day 7 and 10. This is exactly in agreement with Zhongzhi et al.,[59] and Nahla Wahba et al.,[60] who were examined the anti-inflammatory effects of the main constituents of ginger using the same experimental protocol.

Conclusions

The study proved the ability of ginger oil extract to accelerate the healing of induced oral mucositis in rats. This was revealed by oral mucositis score (OMS), histological and immunohistochemical examination of the specimens. The obtained results will help patients that suffering from delaying the ulcer healings especially diabetic and cancer patients for using ginger oil as a topical spray.

DECLARATIONS

Conflict of Interest

No conflict of interest

Contribution of Authors

The authors confirm that this work was completed by the authors listed in this article. The authors declare no competing financial interest**.**

Acknowledgement
The experimental work was performed through the collaboration between National Research (NRC) Centre, Pharmaceutical and Drug Industries Research Institute, Pharmacognosy department for the phytochemical section, Faculty of dentistry, October 6 University (O6U) and Faculty of dentistry, Ain Shams University for biological and histopathological examination.

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