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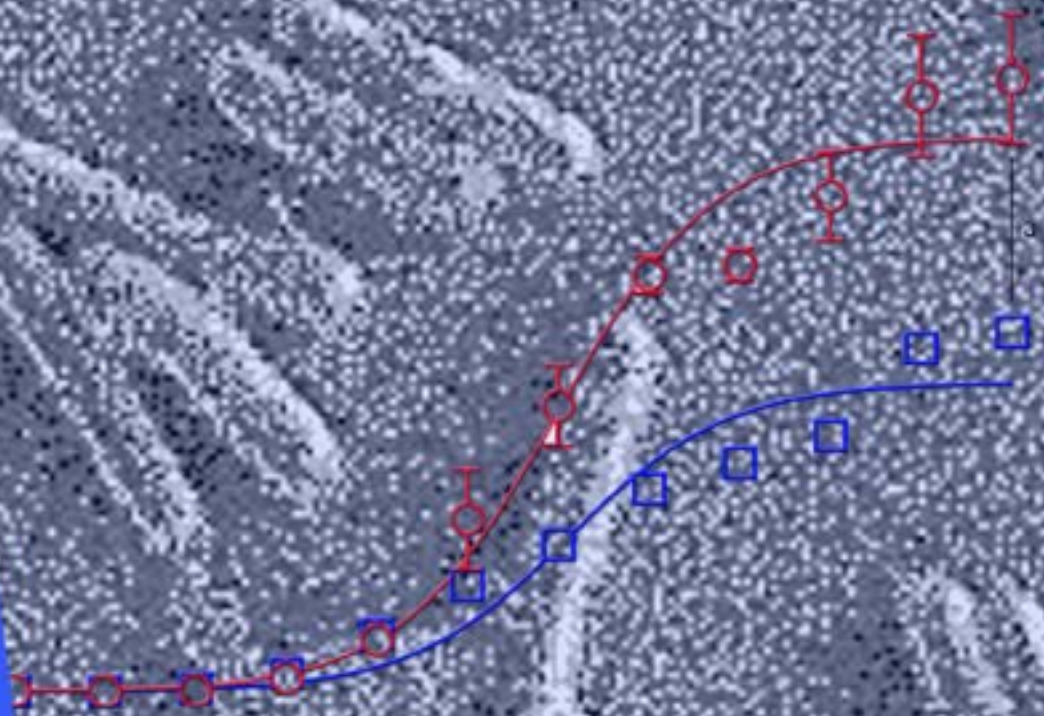

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Contents

The Effects of Combination Between *Bohadschia marmorata vitiensis*, *Eurycoma longifolia, Syzgium aromaticum* Extracts and Ketoconazole Against *Candida albicans*

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ABSTRACT

The crude methanolic extracts of *Bohadschia marmorata vitiensis* (B), *Syzgium aromaticum*(C), *Eurycoma longifolia* (TA) and Ketoconazole (K) were tested for antifungal activity by using the agar-well diffusion methods. This study was carried out to determine the antifungal activity and to evaluate the potential of mixing all these crude extracts against growth of *Candida albicans.* The mixed extracts were prepared by adding 20 mg/ml concentration of each crude extract based on the volume ratio of 1:1, 1:2, 2:1, 1:1:1, 1:1:2, 1:2:1 and 2:1:1. Ketoconazole was prepared at 10mg/ml. Single extract of *Bohadschia marmorata vitiensis, Syzgium aromaticum* and Ketoconazole shows positive effects towards the growth of *Candida albicans*. There are reductions in inhibition zone diameters from the combinations of the extracts TA: C, B: C, B: K, B: TA and G: K: TA. The reduction in diameter of inhibition zone indicates that synergism activity is absent and the extracts react antagonistically with each other. Observations of the *Candida albicans* cell under SEM revealed the antifungal effect of *Bohadschia marmorata vitiensis* extract where there are morphological changes in the fungal cell structures where there are hole formation that indicates membrane instability that lead to fungal cell lyses. In CONCLUSION, the mixtures of the extracts are more effective in inhibiting the growth of *Candida albicans* compared to single extracts.

Keywords: Antifungal, extract, *Bohadschia marmorata vitiensis, Syzgium aromaticum* , Ketoconazole, *Candida albicans*.

INTRODUCTION

There are about 35,000 species of plants used to treat human ailments and therapeutically used as medicine (Lewington, 1993), such as Tongkat Ali (*Eurycoma longifolia*) (Zakaria, 2009) and clove (*Syzgium aromaticum*) (Nassar *et al.,* 2007). Besides plant, animal resources are also use as traditional medicine. In Malaysia, there are about 45 identified species of sea cucumber recognized and used traditionally to treat wound, eczema, arthritis and hypertension (Ridzwan, 2007). *Bohadschia marmorata vitiensis* is one of the recognized species of the sea cucumber. The sea cucumber species also exhibit antimicrobial (Toral-Granda, 2006), antifungal (Hawa *et al.,* 1999), anti-fatigue effects (Wang, 2010), anti-inflammatory and analgesic effects (Villasin & Pomory, 2000).

Eurycoma longifolia is also known as Tongkat Ali, have medicinal values and have its own therapeutic properties. The herbs are used as aphrodisiac, anti-malarial properties (Chan *et al.,* 1986; Kardono *et al.,* 1991; Ang *et al.,* 1995), antipyretic activities (Chan *et al.,* 1995) and antiulcer properties (Tada *et al.,* 1991).

Clove is also known as *Syzygium aromaticum*, *Eugenia aromaticum* or *Eugenia caryophyllata* is a popular herb and used in dentistry as anodyne for emergencies (Cai & Wu, 1996; Phyllis & James, 2000) that can sooth toothache temporarily (Ghelardini *et al*., 2001). Clove has its antifungal properties and claimed to be effective against *Candida* species (Chaieb *et al*., 2007).

Candida is the normal endogenous flora of human (Perfect & Casadevall, 2006). *Candida albicans* can colonize skin and mucosal surfaces of healthy people and thus occurs commensally in the gastrointestinal tract, oral cavity and vagina, often causing superficial infections (Mavor *et*

al., 2005). Excessive growth of the fungus may results in Candidiasis. Candidiasis infections range from superficial to systemic disease.

This paper will evaluate the laboratory experiment to test the effectiveness of the natural remedies combining with synthetic drugs against *Candida albicans.*

MATERIALS AND METHODS

Sample preparation: The methanolic extracts of *Eurycoma longifolia*, *Syzgium aromaticum* and *Bohadschia marmorata vitiensis* was prepared by Faculty of Health Science, Universiti Kebangsaan Malaysia, Kuala Lumpur. The culture media is prepared by using Sabouraud dextrose agar. Single extraction and mixture of the extracts of *Bohadschia marmorata vitiensis, Eurycoma longifolia*, *Syzgium aromaticum* and *Aromaticum* and Ketoconazole is prepared. The samples are weight to about 0.5 mg and the diluents used in the experiment are 5% of DMSO and 95% of distilled water. It was followed by the microorganism inoculums preparation which is done by spectrophotometer and using 0.5 McFarland standard.

Preparation of Fungus Inoculums: *Candida albicans* was prepared using standard method and the antifungal survival test was done using well diffusion methods to detect the antifungal activity in the extract solution (Perez *et al.,* 1990). Analysis of data was interpreted using one way ANOVA.

Morphology observation using scanning electron microscope (SEM). The glass slide contains the fungal sample was fixed in 2% glutaraldehyde solution for 24 hours. Then, washed with PBS solutions (pH 7.3), 3 times for 10 minutes. The dehydration process was then done using serial alcohol concentrations of 30%, 50%, 70%, 90% and 95% each for 10 minutes, followed by 100% alcohol for 15 minutes (twice). The glass slide was then dried before being fix onto a stub. The stub containing the fungal specimen was coated with gold before being observed under the Hitachi SU-8000 scanning electron microscope (SEM).

RESULTS AND DISCUSSION

The single extract of *B. marmorata vitiensis* gives significant reduction in diameter of inhibition zone between the single extract and the mixture of the extract. The active component of sea cucumber has significantly inhibited the growth of the fungus by altering the fluidity and permeability of cell membranes. The extract of *S. aromaticum* has a mean diameter, inhibition zone of 25.0 mm. The clove extract has significantly inhibited the growth of the fungus, hence it has therapeutic potential and the compounds exert their antifungal activity by targeting sterol biosynthesis (Ahmad *et al.,* 2010).

The single extract of *Eurycoma longifolia* does not gives inhibition effects towards the growth of *Candida albicans* showing that the extract does not have active components to inhibit the growth of *Candida albicans*. The results from the single extract of Ketoconazole gave the mean diameter of inhibition zone of 18.0 mm. The results are parallel with research carried out where there are potent inhibitions of ergosterol biosynthesis in *Candida albicans* by ketoconazole at low concentrations (Van den Bossche *et al.,* 1982).

Mixture of the extracts for the combinations of TA: C (1:2) has the mean diameter of inhibition zone of 25.0 mm. This shows that the active components in clove have neutralized the suppressor effects of *Eurycoma longifolia*. The combinations have the same diameter as the single extract of clove. The combinations of B: C (1:2) has the mean diameter of inhibition zone of 23.0 mm. It shows that sea cucumber has suppressed the antifungal properties of clove. This may happen due to competitive activity between both extracts. The combinations of B: K (1:2) has the

Table 1 The inhibitory effect of the extracts on the growth of *Candida albicans*

Extracts	Mean diameter of inhibitions (mm) on *Candida albicans*	
	Ratio	Diameter (mm)
B. marmorata vitiensis (B)	1	18.0
S. aromaticum (C)	1	25.0
E. longifolia (TA)	1	-
Ketoconazole	1	18.0
TA: C	1:1	22.0
TA: C	1:2	25.0
TA: C	2:1	22.0
B: C	1:1	22.0
B: C	1:2	23.0
B: C	2:1	22.0
TA: K	1:1	-
TA: K	1:2	-
TA: K	2:1	-
B: K	1:1	18.0
B: K	1:2	22.0
B: K	2:1	18.0
C: K	1:1	29.0
C: K	1:2	25.0
C: K	2:1	20.0
B: TA	1:1	11.0
B: TA	1:2	10.0
B: TA	2:1	12.0
B: K : C	1:1: 1	13.0
B: K : C	1:1:2	35.0
B: K : C	1:2:1	32.0
B: K : C	2:1:1	28.0
B: K: TA	1:1: 1	-
B: K: TA	1:1:2	-
B: K: TA	1:2:1	-
B: K: TA	2:1:1	-
K: C: TA	1:1: 1	22.0
K: C: TA	1:1:2	29.0
K: C: TA	1:2:1	30.0
K: C: TA	2:1:1	33.0
B: C: TA	1:1: 1	18.0
B: C: TA	1:1:2	22.0
B: C: TA	1:2:1	23.0
B: C: TA	2:1:1	21.0

Indication:

- : No inhibition zone
B : B. marmorata vitiensis extract (20µg).
TA : E. longifolia extract (20µg).
C : S. aromaticum extract (20µg).
K : Ketoconazole (10µg).

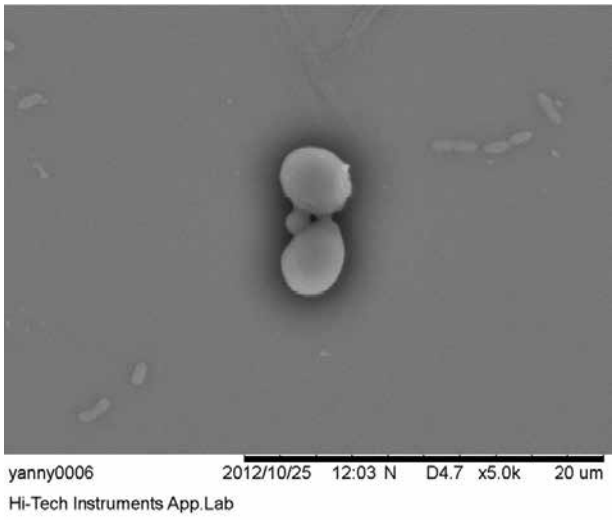

Figure 1: normal cells of *Candida albicans.*

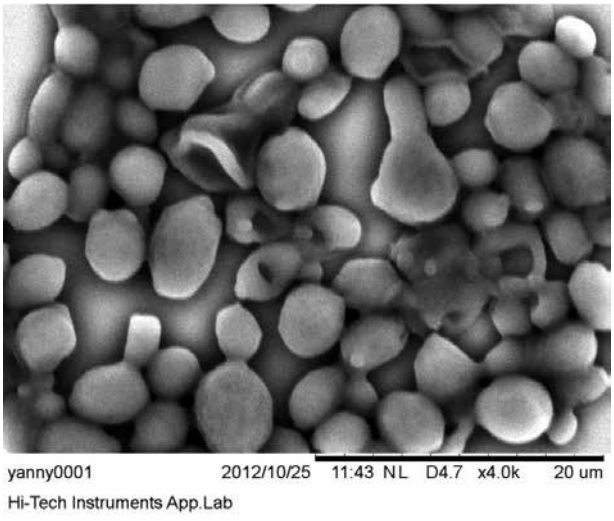

Figure 2 : Effect of ketoconazole on *Candida albicans* **(positive control).**

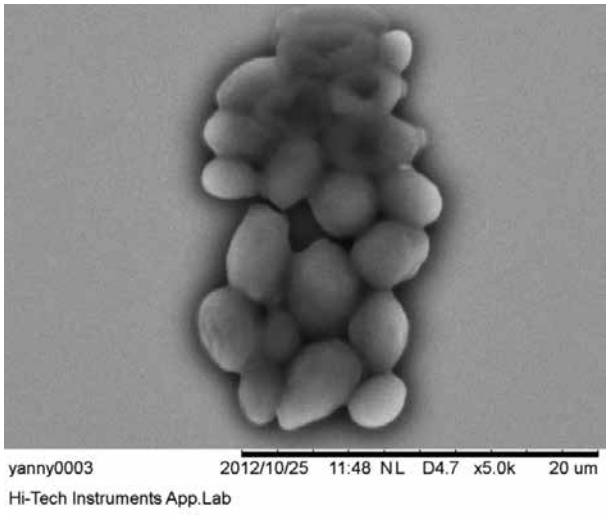

Figure 3: The effect of the mixture of the extracts *B. marmorata vitiensis* **and ketoconazole (1:1) on** *Candida albicans.*

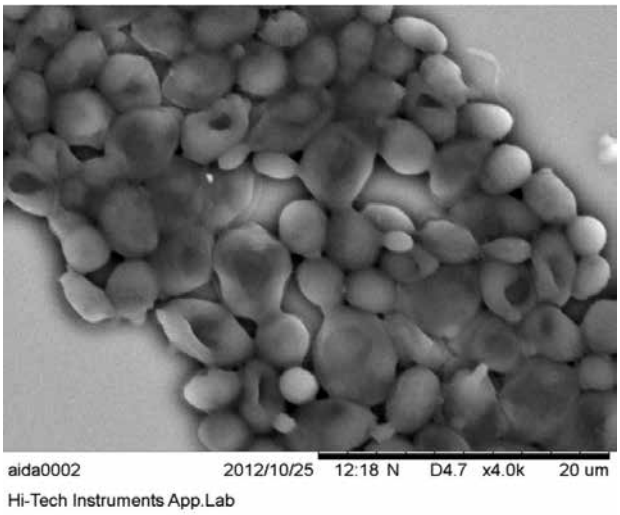

Figure 4: The effect of the mixture of the extracts *B. marmorata vitiensis, clove* **and ketoconazole (1:1:1) on** *Candida albicans.*

mean diameter of inhibition zone of 22.0 mm. The results indicate the enhancement in the effect from the combinations of the extract. The mixture of the extracts are able cooperate to inhibit the growth of the fungus and synergism activity can be observed from the combinations. The mixture of TA: K gives negative effect and unable to inhibit the growth of the fungus. This may happen because *Eurycoma longifolia* suppressed the antifungal properties of Ketoconazole and unable to produce synergism activity was expected. The mixture of C: K (1:1) has the mean diameter of inhibition zone of 29.0 mm. These results shown that clove enhances the effect of Ketoconazole. This supports the finding that the combinations of eugenol and azole will have augmented efficacy and thus have a reduced minimum effective concentrations (Ahmad *et al.,* 2010). Furthermore, the combinations of B: TA (2:1) has the mean diameter of inhibition zone of 12.0 mm. The mixture has lower mean diameter of inhibition zone compared to the mean diameter of inhibition zone of the single extract of *B. marmorata vitiensis* which is 18.0 mm. The extract *Eurycoma longifolia* thus suppressed the antifungal effects of *B. marmorata vitiensis* and synergism activity is absent from the combinations of the extracts.

For the three combinations of the extracts B: K: C (1:1:2) it has the mean diameter of

inhibition zone of 35.0. This combination gives the highest diameter of inhibition. This is due to each of the single extract having positive effect in inhibiting the growth of the fungus, thus the combinations of the extracts having synergistic activity that could enhance the inhibition effect. The combinations have significantly inhibited the growth of the fungus. The combinations of G: K: TA at any combination ratios gave negative effects. This is because the extracts are unable to produce effect together and the combinations suppressed the antifungal properties between each other. Besides that, the combinations of K: C: TA (2:1:1) has the mean diameter of inhibition zone of 33.0 mm and the combinations of B: C: TA (1:2:1) has the mean diameter of inhibition zone of 23.0 mm. From the results, it shows that both of the combinations gave positive results and significant effect in inhibiting the growth of the fungus. The combination ratio has high mean diameter of inhibition and potentiating effect expected from the extract *Eurycoma longifolia* are proven as it enhance the effects compared to the results from the combinations of the extract G: C and K: C.

Observations of the cell morphology using scanning electron microscope (SEM) showed *Candida albicans* cells looks smooth, ovoid, subtle and even size (Figure 1). Ketoconazole affects the cell membrane where the cells are shrinking due to damages on cell membranes and hole formation happens due to cell leakage (Figure 2). The azole antifungal drugs inhibit fungal cell growth by the disruption of normal sterol biosynthesis (Kelly *et al.*, 1995) causing membrane instability leading to leakage of the ion from the cells. The formation of groove and cell bursting from the extract of *B. marmorata vitiensis* is due to the active components in the extracts that may disrupt the membrane integrity of the cells, causing cell damage, changes to the morphology of the cells and results in bursting of the cells (Figure 3). Saponin from the extract has the structure similar to the cholesterol on the membrane and replaces the cholesterol structure thus altering the membrane stability leading to the leakage of the cells (Kaswandi *et al.,* 2007). Saponin has several hemolysis mechanism reported by researcher. Saponin will form complex that is insoluble with cholesterol on cell membrane, and causes either changes in the cholesterol arrangement or those saponin-cholesterol complex that causes disturbance on lipid bilayer (Meyer *et al.,* 1978). This will causes changes in the cell morphology shapes of the cell and causes cell damage (Figure 4). The mixture of the extract of *B. marmorata vitiensis,* and Ketoconazole give similar effects as it blocks the synthesis of ergosterol and reduced the number of ergosterol in the membrane thus causes disruption of the membrane integrity of the cells, causing cell damage, changes in morphology of the cells and lastly results in bursting of the cells.

CONCLUSION

Single extract of *B. marmorata vitiensis, S. aromaticum* and Ketoconazole gave positive effects in inhibiting the growth of *Candida albicans.* The combinations of the extract of K: C: TA (1:2:1) and B: C: TA (1:2:1) gives the largest inhibition zone value which means that it has high potential to inhibit the growth of *Candida albicans*. The potential of *Eurycoma longifolia* to inhibit the growth of microorganisms are proven from the combination ratio of K: C: TA (1:2:1) or B: C: TA (1:2:1). The overall results of the present study showed that combinations of the extract are more effective than single extract in inhibiting the growth of *Candida albicans.*

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Iatrogenic Incidental Ingestion of a Dental Fine Instrument

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ABSTRACT

Ingested foreign bodies are unusual dental scenarioes with potential hazards. These clinically related instrumentation bodies are inclusively ingested via gastrointestinal tract. A case report is briefly written to illustrate management, review and recommendations related to expected on ingested foreign bodies in dental clinic scenario.

Keywords: Iatrogenic, ingestion, restoration, dental bur, radiograph, foreign body aspiration.

INTRODUCTION

Accidental foreign body ingestion and aspiration are among the myriad emergencies that seem to arise in dental clinical seeing. However, the actual documented occurrence of such incidence of accidental ingestion during clinical dental treatment may be under reported. The episodes although are said to be few in occurrence or occasionally, still happens despite being entirely preventable. Regardless of incidence, a foreign body ingestion or aspiration episode are recognized as potential complications in the specialty of orthodontics (Umesan *et al*., 2012) and in management with endodontic instruments (Venkataraghavan *et al*., 2011). *Per se*, fixed orthodontics therapy seems to be identified to have had the highest number of incidents of adverse outcome than aspiration. Dental procedures involving single-tooth cast or prefabricated restorations involving cementation have a higher likelihood of aspiration (Kürkciyan *et al*., 1996). Rhetoric report has indicated that an accidental foreign body ingestion or aspiration incidence involves a (80%) proportion of children below 3 years of age (Aytac *et al*., 1977).

In relation to factors relating to patient positions during the occurrence of the ingestion, Neuhauser suggested that patient in a supine position are more or less prevented from swallowing foreign objects (Neuhauser, W., 1975) while Barkmeier *et al* stated that supine position increase the risk of swallowing (Barkmeier *et al*., 1978). In tandem to this, a lacuna is at present noted that no novel gold standard or rule of thumb guidelines is made available as such whether foreign body ingestion in gastrointestinal tract should be better managed conservatively, endoscopically or thus surgically.

Seven case reports of ingested foreign bodies are presented. Although ingestion of foreign bodies may be a frequent occurrence, 80 per cent of documented ingested foreign bodies pass through the gastrointestinal tract spontaneously (Schwartz, G. and H. Polsky, 1976). The most frequent victims of foreign body ingestion are children, denture-wearing adults, and the mentally ill. Most foreign bodies are best managed by "intelligent neglect" (Schwartz, G. and H. Polsky, 1976). Some require surgical intervention or removal because of presence of perforation, hemorrhage or obstruction. In these clinical scenarioes, the ileocecal region is the most common site for perforation. Close observation for signs of these perforation, hemorrhage, and/or obstruction is of almost mandatory (Schwartz, G. and H. Polsky, 1976). Because many patients who have swallowed foreign bodies are asymptomatic, physicians must maintain a high index of suspicion. The majority of ingested foreign bodies pass spontaneously but serious complications such as, bowel perforation and obstruction can occur. Foreign bodies lodged in the esophagus should be removed endoscopically, but some small, blunt objects may be pulled out using a Foley catheter or pushed into the stomach using bougienage (Bougienage was used as the primary therapy of benign esophageal stricture) (Lanza, F.L. and D.Y. Graham, 1978). Once they are past the esophagus, large

or sharp foreign bodies should be removed if reachable by endoscope. Small, smooth objects and all objects that have passed the duodenal sweep should be managed conservatively by radiographic surveillance and inspection of stool. Endoscopic or surgical intervention is indicated if significant symptoms develop or if the object fails to progress through the gastrointestinal tract (Uyemura, M.C., 2005).

Tamura, *et al.,*(1996) in a review reported a percentage range being 3.6-27.7 % for the finding of ingested foreign bodies (Tamura *et al*., 1986). Ingested objects of dental origin include dental bridges, transpalatal arch, crowns and removable dentures both partial and full, dental floss, bands, impression materials, orthodontic arch wires, retention appliances and various endodontic instruments like broaches, files and reamers (Dibiase *et al*., 2000). As dropping of dental bur may be because of damage of turbine or handpiece cartridge so, some reports declared that while autoclaving handpieces is an essential component of current infection control practices, repeated autoclaving over time can result in deterioration and degradation of the turbine and other parts; the most recent generation of handpieces, however, is more autoclave-resistant than handpieces available in the early 1990s (Little *et al*., 2009). A recent study investigated the potential for high-speed air-driven handpiece degradation following repeated sterilization after simulated clinical use. The handpieces were assessed for a number of mechanical parameters including power, speed, noise, eccentricity and chuck performance after up to 1,000 simulated uses and sterilizations. The study concluded that increased numbers of cycles resulted in increased eccentricity and that the evaluated handpieces would be effective for at least 500 cycles. A second study however revealed that when handpiece ball bearings was assessed, the researchers found no degradation features after a series of 300 autoclave cycles production on the related handpieces studied (Little *et al*., 2009).

CASE REPORT

A healthy 34-year-old malay female patient has attended mobile dental clinic for dental treatment as a regular check-up. On clinical examination found that upper left lateral incusor tooth has incisal dental caries extends meio-distally and no pain on probing, no pain on percussion, no discoloration and past history of no pain at that side.

Past medical History: Patient claimed that she has no history of medical problems.

Past Dental History: Cavity fillings, extraction and regular dental check-up.

Clinical findings:
1) Class II malocclusion with severe crowding of upper and lower anterior teeth.
2) Multiple classes of amalgam fillings.
3) Poor oral hygiene and high caries index.
4) Decayed incisal edge of upper left incisor tooth.

Treatment Plan: For filling of upper left incisor tooth.

A cavity was prepared under copious spray of water as a coolant to prevent thermal generation following general principles of cavity preparation (outline form, removal of decay, resistance form, retention form and toilet of cavity). Isolation of cavity by cotton rolls labially and gauze palatally to avoid saliva and fluids contamination. Acid etching by phosphoric acid, toilet, dryness, bonding agent, curing appication of composite and curing done. Trimming of excess composite incisally, labially and palatally done by tapered stone with pointed end and polishing of labial aspect done by polishing kit. As the operator do finishing of restoration, the fine stone came out and dropped in patient mouth, the operator tried to grip the bur by tweezer but failed due to heavy fluids in the

floor of the mouth, patient been asked to turn to left side and spit the bur but also failed. Patient did not have any symptoms of cough or respiratory distress. Immediately ambulance been called to pick up patient to the nearest radiology center for postero-anterior view of chest and antero-posterior abdominal radiographs. Radiologist have checked the radiograph, the chest radiograph was anatomically normal and the bur was seen on the abdominal radiographic image. The bur was in the stomach in a semi-horizontal position, the shank facing medial wall of the stomach and blades facing lateral wall of the stomach (Figure 1). The patient was admitted to monitor vital signs and symptoms, no any symptoms of colic or abdominal distress. Patient was referred to a

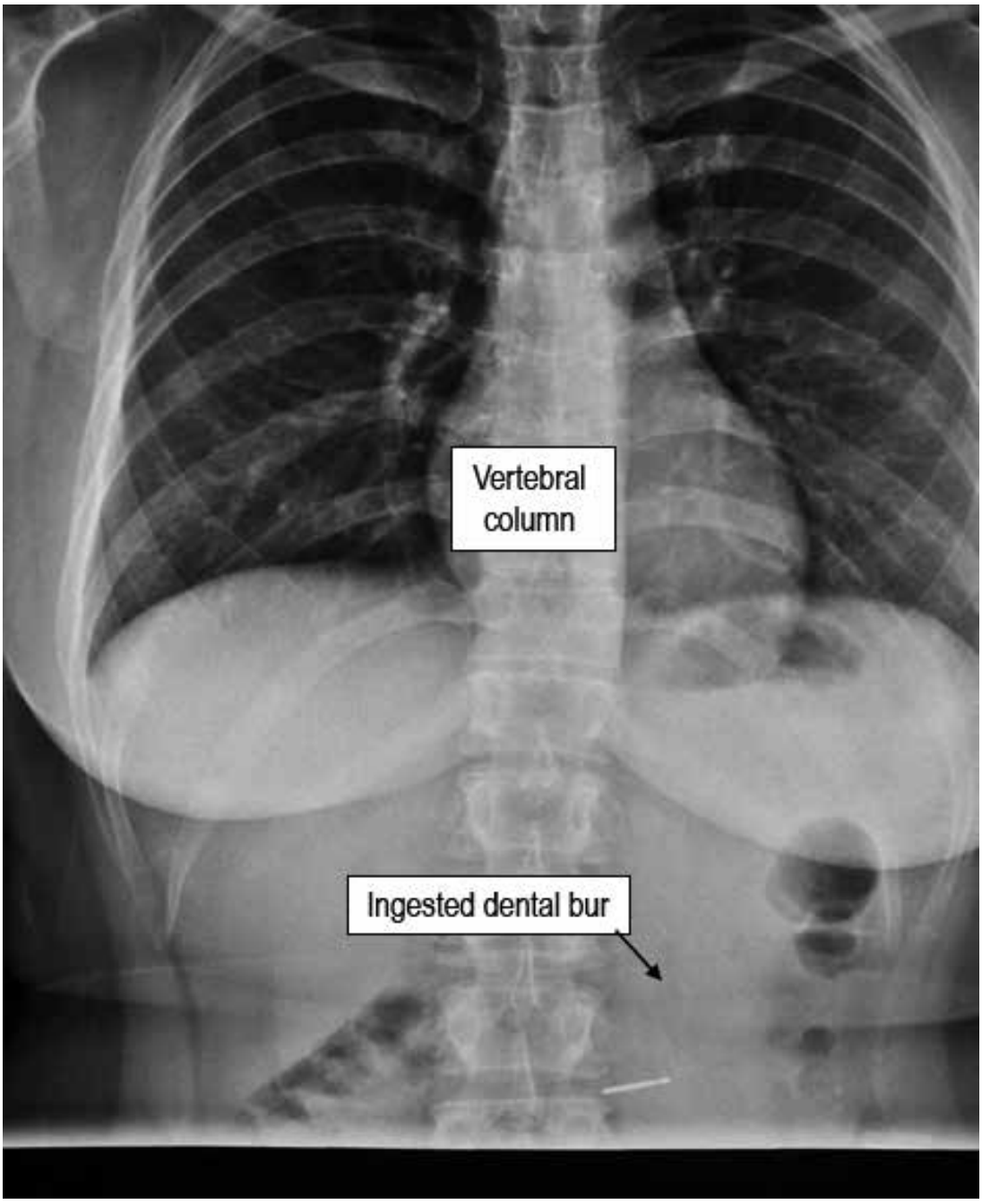

Figure 1: PA radiographic image showing the ingested dental bur located here within the stomach.

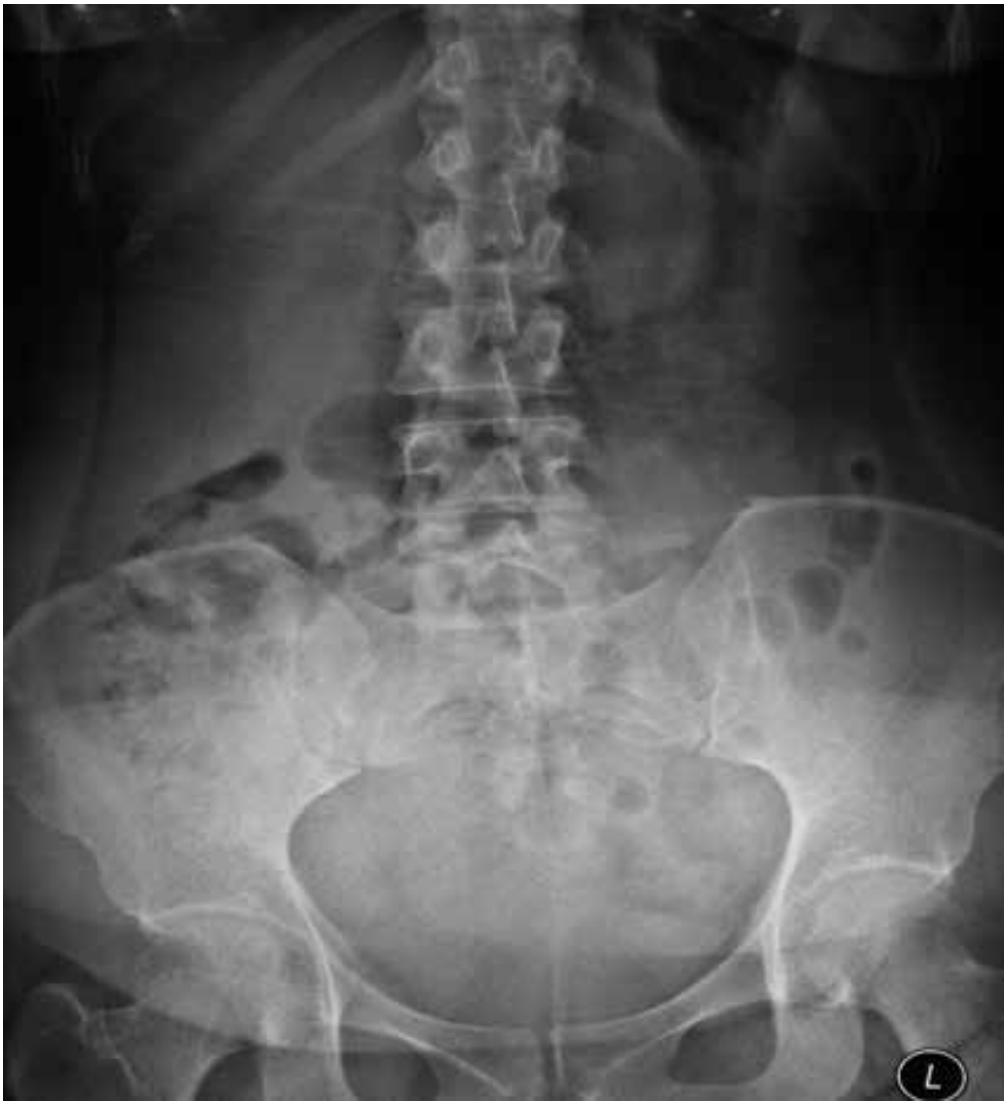

Figure 2: PA radiographic image (pelvic) 3 days post management, showing that the stomach, small and large intestines are anatomically free of any presence of dental bur.

gastroenterologist, he was advised to diet on fibrous food for the next 24 hours, was prescribed laxatives and was advised to inspect her stool regularly.

After 3 days patient re-attended the radiographic center for another antero-posterior radiographic investigation and from that radiographic investigation found that the stomach was free of any foreign body presence (Figure 2).

DISCUSSION

Cases of accidental ingestion or aspiration of foreign bodies during dental procedures are rare, but the potential consequences can be very serious for the patients involved. Accidental ingestion is more common than aspiration, and usually does not cause any clinical signs or symptoms. 90% the foreign objects being egested after passage through the gastrointestinal tract without complication, 10% to 20% necessitate endoscopic removal, whereas only 1% of them will finally need surgical intervention. In clinical practice, we often face the dilemma of choosing the appropriate treatment modality (Kay, M. and R. Wyllie, 2005; Maleki, M. and W.E. Evans, 1970; Bhatnagar *et al*., 2011). There may be complications such as intestinal obstruction, perforation with subsequent abcess formation, hemorrhage, fistulas, or failure of the objects to progress through the gastrointestinal tract (Maleki, M. and W.E. Evans, 1970). Gastric erosion and perforation of the esophagus caused

by ingestion of dental foreign objects have been reported (Athanassiadi *et al*., 2002).

During routine dental treatment, any patient can accidentally swallow or aspirate foreign body or foreign material like filling material, extracted tooth, crown dental bur or endodontic file usually in children (80%) (Pavlidis *et al*., 2008). It is highly risky in physically and mentally disabled children, elderly people and patients under sedation, narcotics or nitrous oxide due to diminished protective reflexes (Ulusoy, M. and S. Toksavul, 2003). It is also reported in some cases of Alzehiemer's disease and Parkinson's disease (Obinata *et al*., 2011). Elderly patients may manifest impairment of sensory and motor nerve responses, which could result in deterioration or dysfunction of the gag/cough reflex. Patients with cerebrovascular diseases may be subject to involuntary movements, and patients with oral cancer who have undergone surgery may have morphological or functional morbidity. In such patients, once foreign objects fall into the oropharynx, they can be easily ingested or aspirated. It has been reported that prisoners, psychotic individuals, alcoholics, patients who are nervous or restless, and patients with an excessive gag reflex are at high risk of swallowing foreign objects (Zitzmann *et al*., 1999). In addition, patients who wear complete dentures ascribed to reduced tactile sensitivity of the palatal mucosa, patients in whom some sites are difficult to access secondary to anatomical restrictions (e.g., a small oral cavity, short palate, macroglossia, or large neck) and those who are barrel-chested, obese or pregnant, in whom increased intra-abdominal pressure are likely at great risk of ingesting or aspirating foreign objects (Zitzmann *et al*., 1999). It is also thought that ingestion or aspiration of foreign objects tends to occur more often in patients with impaired central nervous system function, which can be influenced by medication with sedatives, tranquilizers, opiates, or depressants (Zitzmann *et al*., 1999). Treated teeth are less common and the reason for this appears to be related to the anatomical properties of tooth alignment. Lower molars are closest to the pharyngeal cavity and objects being manipulated in this area may easily be lost. In the treatment of upper molars and incisors, patients are generally forced to lie in a horizontal supine position, which may make it easier for dental objects or instruments to tumble across the tongue dorsum into the pharynx. When treating the lower incisors, fallen or dropped dental objects or instruments would tend to be caught in the oral floor but gag reflex and forces can be the final protective mechanism (Obinata *et al*., 2011).

The complications of foreign body aspiration (FBA) can be divided in two groups: complications related to the foreign body itself and complications following the bronchoscopic procedure. Foreign body aspiration and its evolution can lead to complications such as pneumo-mediastinum, pneumothorax, hydropneumothorax, bronchial stenosis, abscess, atelectasis, pneumonia, bronchiectasis, foreign body dislodgment, and bronchospasm. The presence of these complications in children is about 22% to 33%, and the most common is pneumonia (Oliveira *et al*., 2002).

In tandem to these, via judicious use of airway protection techniques, dental practitioners can avoid the problems encountered by the patient and the doctor. The use of rubber dam with proper precautions is an effective method of preventing aspiration so, it is less common in endodontic cases because of using rubber dam. In procedures that initiates under General Anesthesia (GA) or Deep Nitrous Oxide Sedation (DNOS), the use of protective gauze throat barrier is mandatory (Cameron *et al*., 1996). The first step in managing such cases is accurate determination of the location of object ingested by use of radiographs, ultrasound or magnetic resonance imaging for the best method of intervention considering age of patient and sharpness of the object. 80%-90% of cases pass through the body without complications but in case of delay for days or weeks it means that perforation or obstruction occurred (Webb, W.A., 1995).

The most common noted dentally related objects to be ingested or aspirated in dental practice are metal inlays, metal cores, metal crowns.The reason for this may be that some restorative or prosthetic objects are small andslippery, making them difficult to handle manually. Once a foreign object has reached the stomach, there is a greater than 90% chance that it will pass through the gastrointestinal tract as a result of peristaltic movement without complications, usually after a 7-10-day period (Webb, W.A., 1995). As a precaution, it is recommended that swallowed foreign objects

be assessed by serial radiography until egested. If patients develop symptoms of perforation, such as pain or vomiting, tenderness or abdominal guarding, and if objects remain lodged longer than 2 weeks, surgical intervention is required.

Aspiration always requires immediate treatment, as in this setting foreign objects can cause inflammatory reactions or even severe obstruction leading to death (Ayed *et al*., 2003). If a foreign object is lost into the oropharynx, the patient should be placed in a reclining position, and encouraged to cough vigorously to secure the airway. It is thought that foreign objects are likely to fall into the right bronchial tree because it is oriented more vertically, and in fact clinical data show that such objects become preferentially localized on the right side (Ülkü *et al*., 2005). Symptoms such as choking, inspiratory stridor and labored breathing are signs of airway obstruction by an aspirated foreign objects (Heimlich, H.J., 1977). If further vigorous coughing fails to bring improvement, the Heimlich maneuver should be performed, and attempts made to relieve the laryngeal obstruction (Heimlich, H.J., 1977). This procedure needs to be performed as soon as possible after aspiration, otherwise emergency help must be summoned immediately for transfer of the patient to a hospital emergency unit. Whilst awaiting help, the practitioner and his/her team must consider measures for emergency life support, including airway provision via a cricothyroidotomy, if appropriate and feasible (Milton *et al*., 2001). The security manual issued at our institution stipulates that the first step is to immediately call for help from any surrounding personnel, because the practitioner involved in this kind of event is often flustered, and then an emergency team, including an anesthesist, should be called. However in case of aspiration or ingestion of an object, plain x-ray should be taken and in some cases like impression materials or resins which are made of substances that lack radiopacity, making it impossible to identify their position by X-ray, and therefore diagnostic bronchoscopy or computed tomography is necessary for their localization (Obinata *et al*., 2011).

Per se, considering the risk chances of life-threatening emergencies related to these accidental aspiration and ingestion, dentists must ensure meticulous precautions and be ready to deal with such emergency during dental procedures (Obinata *et al*., 2011). If a clinician happens to face such an emergency, the first active step would be not to panic but to carry out immediate management procedures in a systematic manner; such as, finger sweep method, back blows, chest thrust or Heimlich maneuver for retrieval of foreign body or rescue breathing and calling for emergency medical services if the patient is unconscious or not responding. This effort can be followed either by systematic investigations or opinions regarding intervention (Amarlal *et al*., 2009).

Literature review recommendations for top management of clinical scenario of ingested/ aspirated dental instrumentation pieces. Dental practitioners with careers shorter than 5 years were more likely to allow accidental ingestion to occur. However, even very experienced dental practitioners can also make mistakes in this respect, suggesting the importance of instituting precautions and countermeasures at all times. The majority of the reported literature describes the management of ingested blunt objects. However, ingestion of sharp objects can still occur with a higher rate of perforation corresponding to treatment dilemmas. Urgent endoscopic assessment and retrieval of recently ingested sharp dental foreign body is indicated (Bhatnagar *et al*., 2011). A number of methods for preventing aspiration or ingestion have been described. Endodontists encourage the use of a rubber dam during endodontic procedures, both for prevention of ingestion or aspiration as well as for reducing stress arising from safety concerns and improved infection control (Bhatnagar *et al*., 2011; Susini *et al*., 2007). Other methods including throat packs (gauze throat screens) and retaining ligatures have been advocated. However, these methods may be uncomfortable for the patients and cannot be used at times of occlusal adjustment. A technique using dental floss that is instantly glued to fixed restorations or knotted to posts and cores has been shown to be simple and cost-effective (Al-Rashed, M.A., 2004; Nakajima, M. and Y. Sato, 2004). In the treatment of molars, the present authors suggest inclining the head of the patient to one side to help catch objects in the buccal pouch. However, the best countermeasure is still meticulous care to fix burs tightly and to use dental instruments in the properly prescribed way. Additionally,

practitioners can make patients aware of the possibility of dental objects dropping in such cases, and instruct them to spit out any dropped objects. Obviously, there is also a need to organize smooth support and cooperative procedures that can be implemented promptly if accidental ingestion or aspiration occurs.

To reduce the risk of turbine degradation, the manufacturer's instructions for cleaning, lubricating and sterilizing the handpiece must be followed. A number of techniques are available for cleaning and lubricating prior to autoclaving. Certain cleaning solutions and foam sprays are available that are introduced into the inner area of the handpiece. Debris must be removed, the inner surfaces cleaned, and the handpiece lubricated. Depending on the spray selected and the manufacturer's instructions, lubrication after autoclaving may or may not be required. In addition to manual cleaning and lubricating, automated machines are available with short cycles of automated cleaning and lubricating.Another automated system has a 35-second cycle time and delivers cleaning solution through air/water lines, as well as oiling turbines and chucks prior to sterilization (Little *et al*., 2009).

Finally, failure to clean handpieces prior to sterilization results in a failure to sterilize the handpiece due to the presence of debris, and also clogs chuck mechanisms and turbines. Failure to lubricate handpieces with the exception of lube-free handpieces, which do not require lubrication contributes significantly to early bearing failure (Little *et al*., 2009). Handpieces must be sterilized following cleaning and lubrication. If a handpiece cannot be heat sterilized, it should be safely and permanently discarded (Rutala *et al*., 2008). Heat sterilization methods include the use of dry heat, chemical vapor or, most commonly, the pressurized steam of an autoclave. The Centres for Disease Control and Prevention (CDC) guidelines recommend autoclaving of handpieces (steam sterilization). The instructions from both the handpiece manufacturer and the autoclave manufacturer must be followed to avoid potential damage to the handpiece and to ensure that the sterilization cycle is effective (Little *et al*., 2009).

Guidelines and precautions with removable appliances:

1. All metal retentive components should be inspected at every appointment for any sign of fracture due to repeated wear. Refabrication of the appliance is indicated if this is observed.

2. The acrylic plate(s) should be inspected for cracks due to crazing or thinned-out areas, especially on load-bearing surfaces to preclude accidental damage to appliance during use.

3. It is recommended that the acrylic used to fabricate the appliance be preferably radio-opaque. This will facilitate easier localization in the event of ingestion of the appliance or part (s) thereof.

CONCLUSION

In conclusion, the previous reported scenarioes do have potential emergencies. However with proper guidelines, such incidents can be minimized or uneventful.

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Modified Uranyl Acetate Replacement Staining Protocol for Kidney Ultrasections

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ABSTRACT

A simple modification in the staining protocol of Uranyl Acetate Replacement for kidney sections is described. This preparation is safe, faster and gives good contrast compared to using Uranyl Acetate staining protocol in resin kidney tissue sections. The modified staining protocol of Uranyl Acetate Replacement also reduces charging effects that were seen in the original Uranyl Acetate Replacement staining protocol. This modified staining protocol of Uranyl Acetate Replacement is relatively much safer because it contains no radioactive substances in normal usage in electron microscopy laboratory.

INTRODUCTION

In electron microscopy, images are really no more than magnified projections of the various densities proportionate to the components of the section. In order to achieve a differential increase of the densities in biological structures, differential contrast is needed to produce a sharp image definition.

A post staining technique was used to yield a good result that it has become one of the most commonly used techniques for producing the required contrast effect. But it has a drawback in that the uranyl acetate is a radioactive substance that can cause severe health risk for the laboratory staff due to prolonged exposure.

In TEM, electron scattering with the specimen mainly produces contrast. Structures that strongly scatter electrons are referred to as electron dense and appear as dark areas and structures that scatter fewer electrons appear bright (electron transparent) (Flegler *et al*., 1993).

To increase their contrast, electron dense stains can be added to the sample, the most commonly used heavy elements being: gold, platinum, tungsten, lead, and uranium. Biological specimens can be contrasted through various staining techniques: Particles like protein complexes or viruses can be embedded in heavy metal salts (negative staining), or the specimen can be covered with very thin electron-dense metal films (replicas produced by shadowing). Cells and tissues can be infiltrated with stain before embedding (Osmium tetroxide or Uranyl Acetate en bloc staining) or the ultra-thin sections are stained (Dykstra, 1992)..

The most frequently used method for post-staining is a two-step procedure of staining with uranyl acetate, followed by lead citrate. Uranyl Acetate is used as an aqueous solution with a pH for the saturated solution in the range of 3.5 to 4.0. Uranyl acetate strongly stains proteins as well as nucleic acids and phospholipids. When applied after the Uranyl Acetate staining, lead citrate (prepared according to Reynolds, 1963) will increase this contrast (Dykstra, 1992).

Although Uranyl Acetate is an excellent and well-characterized stain, replacements are sought for several reasons. When it needs to be handled as a powder, it is very toxic and carcinogenic if inhaled. Furthermore, depleted Uranyl Acetate is considered a radioactive material, and hence subjected to stringent regulations by the authorities. Therefore, Uranyl Acetate requires safe storage and careful handling, which in turn increases cost for shipping and waste disposal.

The reagent described in this study as replacements for Uranyl Acetate is Uranyl Acetate

Replacement. Only very little information's about Uranyl Acetate Replacement has been published and the methods are not well known in the Electron Microscopy community especially in Malaysia.

Uranyl Acetate Replacement was described as post-stain protocol to electron microscopy by Nakoshi *et al*. (2011) (OUAR), and was prepared from two lanthanide salts, samarium triacetate and gadolinium triacetate. Therefore it was assumed to be harmless for health and environment, even though the supplier failed to produce a material safety data sheet. It can be purchased from Electron Microscopy Society and is delivered as a liquid. In our preliminary study, Uranyl Acetate Replacement reaction with kidney ultrathin sections produces charging effects and reduces contrast and sharpness. Therefore we introduce post-staining of two-step procedure of staining with Uranyl Acetate Replacement, followed by lead citrate (MUAR). We hope to enhance the quality of contrast and sharpness of stained specimens compared to UA.

In this study, we like to introduce a modified staining protocol Uranyl Acetate Replacement in replacing uranyl acetate reagent. Generally Uranyl Acetate Replacement does not produce good images as it produces lots of charging effect. It is mainly used as a negative stain for viruses and bacteria. Charging effects causes electrons to accumulate during staining and affects the quality of the stained specimens. By modifying the Uranyl Acetate Replacement staining protocol, we hope to increase the contrast and sharpness of the electron micrograph and at the same time minimize the risk for the laboratory staff.

MATERIALS AND METHODS

We carried out a manual contrast method and conducted a direct comparison of results from the different post-staining protocol of UA, OUAR and MUAR on kidney samples. The ultrathin sections of the kidney samples that were post stained with UA, OUAR and MUAR protocol were qualitatively analyzed and observed for sharpness, contrast, brightness and charging effect.

Sampling and Preparation of Sample

Freshly dissected mice kidney tissue were fixed in 2.5 % phosphate buffered glutaraldehyde and post-fixed with 2.0% osmium tetroxide. The mice were sourced from Animal House, Institute Medical Research. Mice kidney tissue were dehydrated with 50%, 70%, 90%, 100% (3X) acetone and embedded in Agar 100 epoxy resin and sectioned to a 90 nm thickness. A sample grid is floated, section side down on the stains following the protocols of UA, OUAR and MUAR. After blotting each off the stains, the grid was rinsed thoroughly with distilled water to remove any residual unbound stain and stained with Reynolds' stain (Table 1). The post stained ultrathin sections of samples were viewed under TEM at 100 KW.

Preparation of Reynolds' Stain

1.33 g lead nitrate and 1.76 g sodium citrate were mixed in a 30 ml distilled water and were shaken vigorously. The solution will be a milky white color. 1.0 ml of 1 N sodium hydroxide was added and mix well to clear the solution Then the solution top up to 50 ml distilled water and pH was adjusted to 12.

Preparation of UA stain

I g of Uranyl Acetate were dissolved in 50 ml distilled water.

Preparation of MUAR and OUAR stains

UAR were purchased from Electron Microscopy Sciences, USA. Preparation of the MUAR and OUAR stains as in Table 1.

Table 1: Details of Manual Staining Procedures

Protocol	Time	Washing	Time	Washing
UA	20 min (2% Uranyl Acetate)	ddH_20	20 min (Lead Citrate)	ddH_20
OUAR	30 min (1 part Uranyl Acetate Replacement with 4 part distilled water)	ddH_20	No	
MUAR	5 min (1 part Uranyl Acetate Replacement with 4 part distilled water)	ddH_20	5 min (Lead Citrate)	ddH_20

RESULTS

The images (Figure 1, 2, and 3) using the stain protocols described (Table 1) shows variation in the degree of sharpness, contrast and brightness in the stained kidney sections. The MUAR protocol gives more detailed lines of structures that can be clearly seen compared to other protocols. Differential contrasts between the different biological structures were well defined in MUAR protocol then in other protocols. There were significantly more charging effects on the kidney sections in OUAR compared to other protocols. Sharpness, contrast and brightness between the MUAR and UA protocol were of high quality in MUAR then in UA.

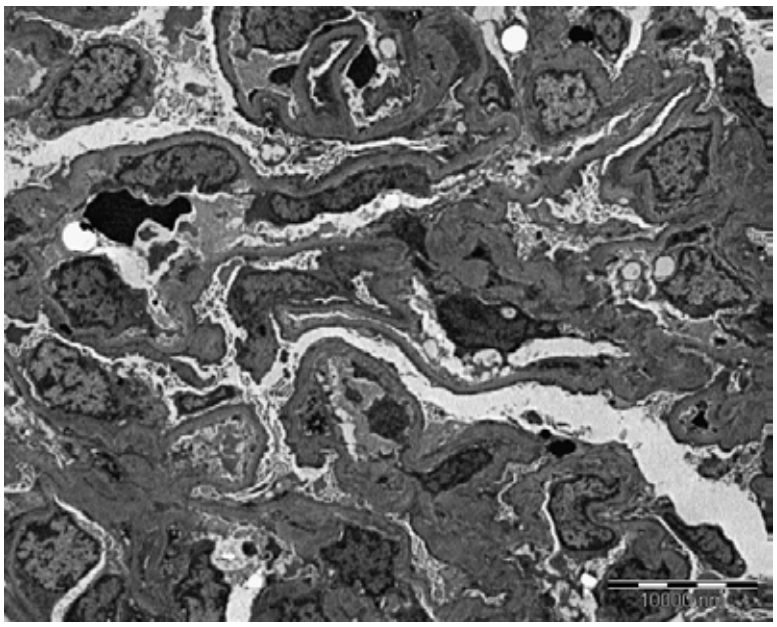

Figure 1: Kidney section stained with MUAR protocol. Sharpness, contrast and brightness were well defined using MUAR compared to OUAR and UA. Magnification at 4500X. Bar 100nm -----.

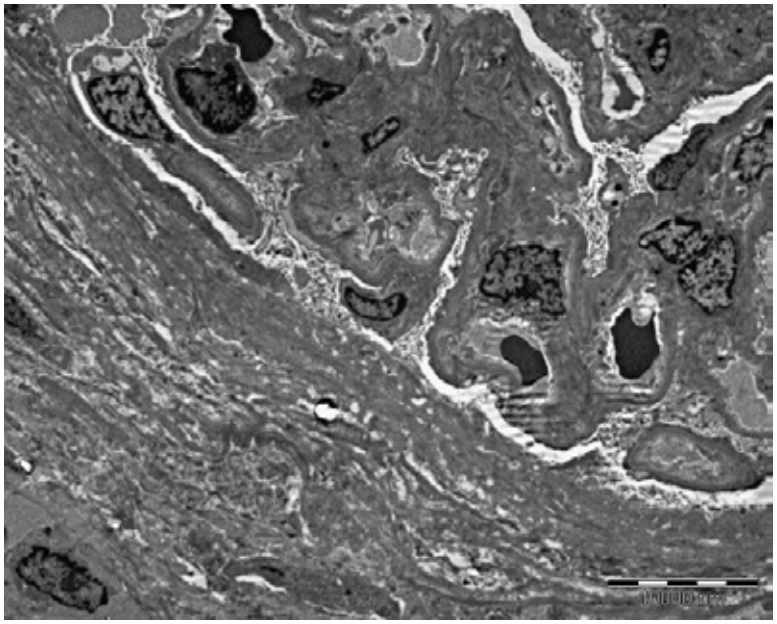

Figure 2: Kidney section stained with UA protocol. Sharpness, contrast and brightness using UA protocol less than MUAR but better then OUAR. Magnification at 4500X. Bar 100nm -----.

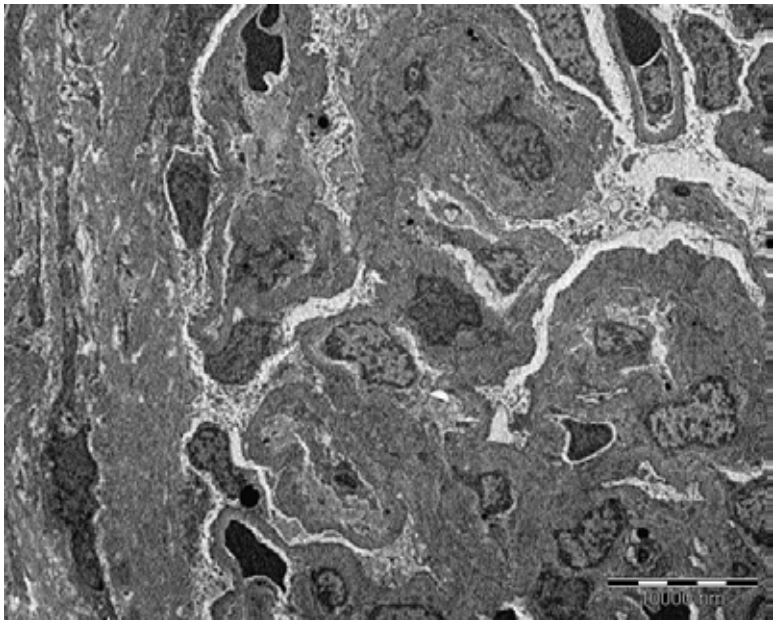

Figure 3: Kidney section stained with OUAR protocol. Charging effect were very obvious compared to MUAR and UA. Magnification at 4500X. Bar 100nm -----.

DISCUSSION

The staining protocol in MUAR is superior compared with OUAR and UA. MUAR produces quality kidney images and provides defined ultrathin structures of the kidney's organelles. Moreover, MUAR needs shorter time to complete compared to other staining protocols (Table 1). The total protocol time for staining kidney sections is less than 15 minutes including the washing steps. MUAR staining protocol provides an alternative staining to the UA staining (as it is a gold standard in staining electron microscopy ultrathin sections) and it is safer to use as did not contain any radioactive substances. The MUAR protocol has been used in our laboratory for about a year. Apart from kidney, other organ tissues and cell cultures have been examined successfully using MUAR staining protocol in our laboratory successfully.

CONCLUSION

The MUAR staining protocol has eliminated the need to use highly toxic radioactive substances thus making the staining protocol safe to use in the electron microscopy laboratory.

The MUAR staining protocol provides good contrast, fast and easy to use.

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Morphological Characterizations and Sensorial Properties of Yeast Bread (Bun) Formulated with Different Particle Sizes of Cornlettes (*Zea mays L.*)

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ABSTRACT

Cornlettes or immature corn which is one of the commonly consumed vegetable by Malaysian populace contains high dietary fiber in dried form. Presently, cornlettes have been introduced in enhancing nutrition-al qualities of baked-based products. This study aims to investigate the influence of different particle size (45, 125 and 250μm) of cornlettes on morphological characterizations of bun. Scanning electron micro-scopically observation showed that at higher magnification, there are compact particles of wheat flour and dietary fibers especially bun formulated with larger particle size of cornlettes. In addition microstructure of cornlettes with oily particles intact were clearly seen. Dietary fiber of cornlettes was seen able to absorb fat molecules. All sensory attributes of bun formulated with CLP were not significantly different with the control bun. Addition of 250μm PS of CLP in bun resulted in yielding softer and elastic bun. In brief, bun added with 250μm particle size of cornlettes is recommended in the preparation of high fiber and palatable bun.

Keywords: Cornlettes, morphological characterization, Scanning electron microscope (SEM), Sensory evaluation

INTRODUCTION

Dietary fiber is a ubiquitous component of plant foods and includes materials of diverse chemical and morphological structure while resistant to the action of human gastrointestinal enzymes. Within the gastrointestinal tracts, fiber forms a matrix with both fibrous and amorphous characteristics. The physicochemical properties of this matrix determine the homeostatic and therapeutic functions of dietary fiber in human nutrition. Fiber swells within the aqueous medium of the intestinal lumen taking up water and small molecules including fats.

Due to insufficient intake of dietary fiber by various communities worldwide, there is significant awareness to increase the intake of dietary fiber in baked-based food items including bun. Generally, the average of usual intake for dietary fiber is low around 16g/day while dietary recommended intakes (DRIs) suggested that the daily consumption of dietary fiber should reaches up to 25 g/day for women and 38g/day for men (Timm and Slavin, 2008).

Low intake of total dietary fiber among men and women globally can lead to increase the prevalence of non-communicable disease such as cardiovascular disease, cancers, chronic respiratory disease and diabetes. Thus by increasing the dietary fiber intake in baked-based products, the dietary fiber levels among healthy individuals in the society will be improved. Besides, there are various baked-based products available on the shelf today which are not favourable in texture and might be influenced by the ingredients used. Many studies have been done to include different plant-based materials in baked-based products but it led to harder texture, and loss it nutritional and functional values. Particle size of additive ingredients like barley, oat, corn and other plant based materials have significant effect on baking potential and final quality of finished product in bakery (Zhang *et al*., 2005).

The present study aims to investigate the morphological profiles and sensory properties of different particle sizes of cornlettes added in yeast bread (bun).

MATERIALS AND METHODS

Preparation of cornlettes powder

The vegetable cornlettes were bought from Pasir Mas district of Kota Bharu, Kelantan state of Peninsular Malaysia. The cornlettes were dehusked manually. The cobs were manually separated from cornsilk and washed with distilled water. The fresh cornlettes were then manually chopped into small pieces, and air dried for 12 hours on stainless steel tray with circulating fan. After that, the dehusked cornlettes were oven dried at 55^0C for 2 days until yellowish threads were obtained. The yellowish dried cornlettes was then ground into pow-dered from using electrical grinder (National Brand, MX-895, Malaysia) and cornlettes powder was then sieved by using analytical siever (AS 200, Germany) having diameter of 45µm, 125µm and 250µm.

Formulation for bun using different particle size (PS) of cornlettes powder (CLP)

The ingredients used in bun preparation were dry yeast, fresh milk, butter, sugar, salt, egg, water, wheat flour and cornlettes powder according to Lim and Wan Rosli (2013). The bun was prepared by adding 4% of CLP to partially replace wheat flour (4% CLP + 96% wheat f1our) at different particle size of cornlettes (45µm, 125µm and 250µm).Bun containing 100% wheat f1our was used as a control.

For preparation of the bun, there were several steps involved. Firstly, flour and instant yeast were mixed evenly. Then, the water was stirred into flour mixture and kneaded for 5 minutes. The sugar, salt, egg, butter and milk were stirred and kneaded until smooth and soft.

The dough was then rest for 20 minutes. The CLP was mixed in the dough and allowed to rest for 15-25 minutes. The dough was then divided each of them was weighed for 60g and let to rest again for another 10-15 minutes. The dough was then rolled into the round shape and placed into the pan which pre-brushed with parchment of butter. The dough was covered with humid towel and rise in warm a place (35^0C) until it doubled its size in about 1 hour. Finally it was baked at 1500C for 15-20 minutes or until done.

Characterization of bun added with different particle sizes (PS) of cornlettes powder (CLP)

The bun samples with different level of PSs were cut into smaller cubicle size (4-5mm in diameter) as the aluminum stub was so small (less than 1 cm). The samples were then placed on aluminum stub and are coated with 99.99% pure gold by sputter coating machine (Leica, SSD 005). The samples on stub surface where coated with a thin layer of gold (less than 30 nm thickness). Then carefully transferred the samples to SEM machine (FER, Netherland, Belanda). The results later were compared among different PSs of CLP in bun. The microstructure of bun was viewed and interpreted.

Sensory evaluation of bun added with different PSs of CLP

The sensory evaluation of the bun formulated with different PSs of CLP was conducted using 30 untrained panelists from students and staffs of the School of Health Sciences, Universiti Sains Malaysia. The parameters evaluated by the panelists included color aroma, softness, elastic, flavor and overall acceptance. Bun samples were evaluated for above mention criterion on a seven-point hedonic scale ranging from 1 (dislike the most) to 7 (like the most).

RESULTS AND DISCUSSION

At 500X magnification, the elongated dietary fiber of cross-sectional bun added with 45µm particle size of cornlettes was clearly observed. The straw like-structure of cornlettes fiber thread is attached to the gelatinized starch granules and other ingredients (Figure 1b). The following photomicrographs (Figure 1c and 1d) show irregular features of cornlettes fiber which are sticked/ attached to the cross-sectional surface of bun. There is possibility of cornlettes fiber to expand

during the process of bun-making. Both fermentation and proofing process undergone by the bun dough may allow to the dietary fiber to absorb water and oil molecules and disturbing the hydration of starch and protein. This situation may explain why some of the dietary fiber was expanded to a slightly longer size (from the original size of 125 and 250 μm). In addition, majority of cornlettes fibers are insoluble in water (Fakurudin *et al.* 2013). Increasing the content of insoluble fiber reduces the sectional expansion of extruded cereals (Robin *et al*., 2012).

Dietary fiber are differs from each other from their solubility, which most of them is insoluble in water. Photomicrographs which show the prominent fiber thread of cornlettes covered by fat is insoluble dietary fiber type (Figure lc and ld). The effect of fiber type on expansion volumes often depends on the process conditions. For instance increasing wheat bran from 0 to 20% content has been shown to have only a limited effect on the sectional expansion of an extruded wheat flour/ pinto flour bean blend at high water content in the extruder (Hernandez-Daz *et al*., 2007). On the other hand, at low moisture content, increasing fiber of wheat bran significantly decreases the sectional expansion. The decrease in sectional expansion when increasing insoluble dietary fiber content often leads to an increase in longitudinal expansion (Lue *et al*., 1990, 1991; Jin *et al*., 1995; Robin *et al*., 2011a; Stojecska *et al*., 2008b). So, the insolubility of dietary fiber affects the expansion rate of cornlettes fiber thread during bun making. In addition, some of the other dietary fiber threads are either shrived or maintained its original structure within gelatinized starch granules.

The shriven of fiber thread may possibly be due to the contribution of physical force that able to destroy or partially destroy the original structure of raw ingredients include cornlettes. This physical force during dough mixing perhaps leads to the breakage of fiber thread. Some of them are seen being entrapped with other ingredients or starch granules. The unchanged and unaffected morphological structure of some cornlettes fiber may be minimally exposed to the mixing blade during dough mixing process.

At 2000X magnification, the irregular shape of partially gelatinized starch granules and translucent fat ingredients from butter are clearly seen in Figure 2a. Partiality broken cornlettes fiber thread embedded within the bun texture is clearly observed (Figure 2b). The fiber thread is broken perhaps due to increase exposure toward mixing blade during dough preparation of bun making. The partially broken cornlettes fiber thread with shining oil or fat covered on it is seen trapped in the gelatinized starch-gluten structure (Figure 2c). Insoluble dietary fiber type of cornlettes has been identified as oil absorbent which it may retain the fat in bun making. This action is completed by its characteristic of capillary attraction. When mixing of the dough or during fermentation, some of the fiber threads are broken or partially broken but their functionality to absorb the oil is still occurred (Figure 2b and 2d).

The present results are in agreement with the study conducted by Fakurudin *et al.* (2013) who studied the morphological characterization of dried cornlettes. There are the analogous structure of single pith in the cortex cell wall. Pith or multi spongy substances are prominently found in the stems of dried cornlettes. The present study revealed that the morphological features of processed cornlettes are minimally affected by the mechanical forces during dough mixing, kneading, fermentation and proofing prior to baking. Baking of proofed dough was seen to be not affected or minimally affected the integrity of cornlettes fiber. This situation may explain the reason why some of the cornlettes fibers are still adhered or embedded in the gelatinized starch-gluten baked bun structure.

Sensory evaluation test of bun formulated with different PS of CLP

The development of acceptable sensory characteristics of CLP bun with high dietary fiber is important to improve the quality of finished product. Commonly, characteristics of good quality of CLP buns are flavourful, golden in color, soft, elasticity and good in appearance. In general, bun should be soft and tender inside, with a good crumb. The crust should be evenly convex and golden brown or a bit darker (Megas, 2013).

Table 1 shows the mean score for sensorial acceptance of bun formulated with different PS of CLP and control. AII sensory attributes were not significantly different ($P > 0.05$) for all bun formulated with different PS of CLP and bun formulated without CLP (control) except for color. Panelists like the most of control bun in term of overall acceptance (5.13), color (5.43) and flavour (5.00) while they like the most CLP bun formulated with 250µm PS in term of softness (5.33) and elasticity (4.43). However, bun formulated with 45µm PS of CLP recorded the highest score (4.50) for aroma attribute.

In addition, control bun recorded the lowest value of aroma (4.20), while CLP bun added with 45µm PS recorded the lowest likeness of overall acceptance (4.57), elasticity (3.97) and flavour (4.23). CLP bun formulated with 125µm PS recorded the lowest value of color (4.30) and softness (4.30). However, the scores of all sensory attributes of the CLP- based bun were still at acceptable range scores.

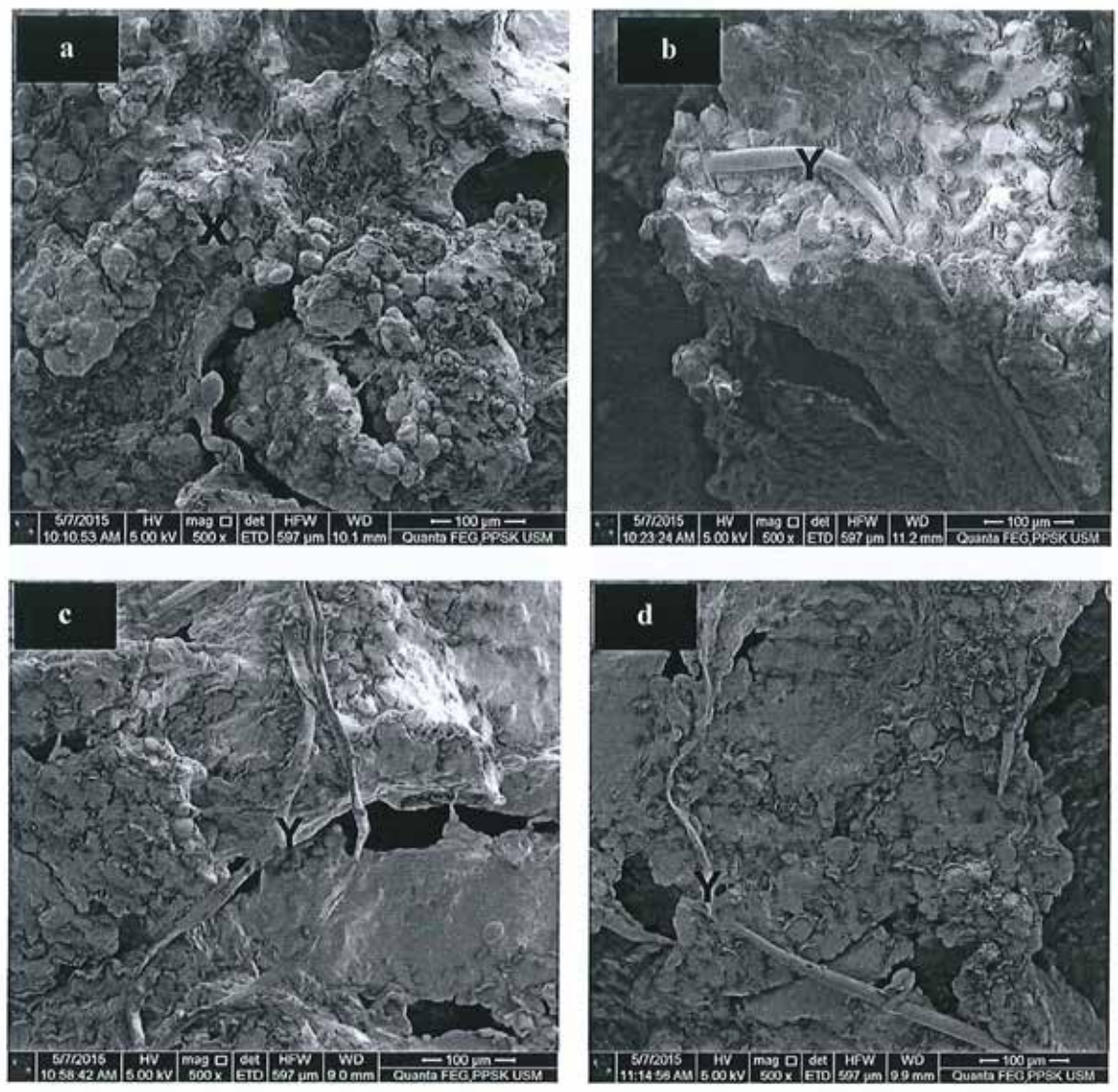

Figure 1. Scanning electron photomigrograph of bun samples (500X magnification) containing different particle sizes (PS) of cornlettes powder (CLP). 1a: bun sample containing 0% of cornlettes (control) 1b: bun con-taining 4% with 45µm PS of CLP, 1c: bun containing 4% with 125µm PS of CLP, 1d: bun sample containing 4% with 250µm PS of CLP. Photomicrographs of 1b, 1c and 1d show elongated dietary fiber (Y) of cornlettes which were glued together with starch granules (X) and other ingredients.

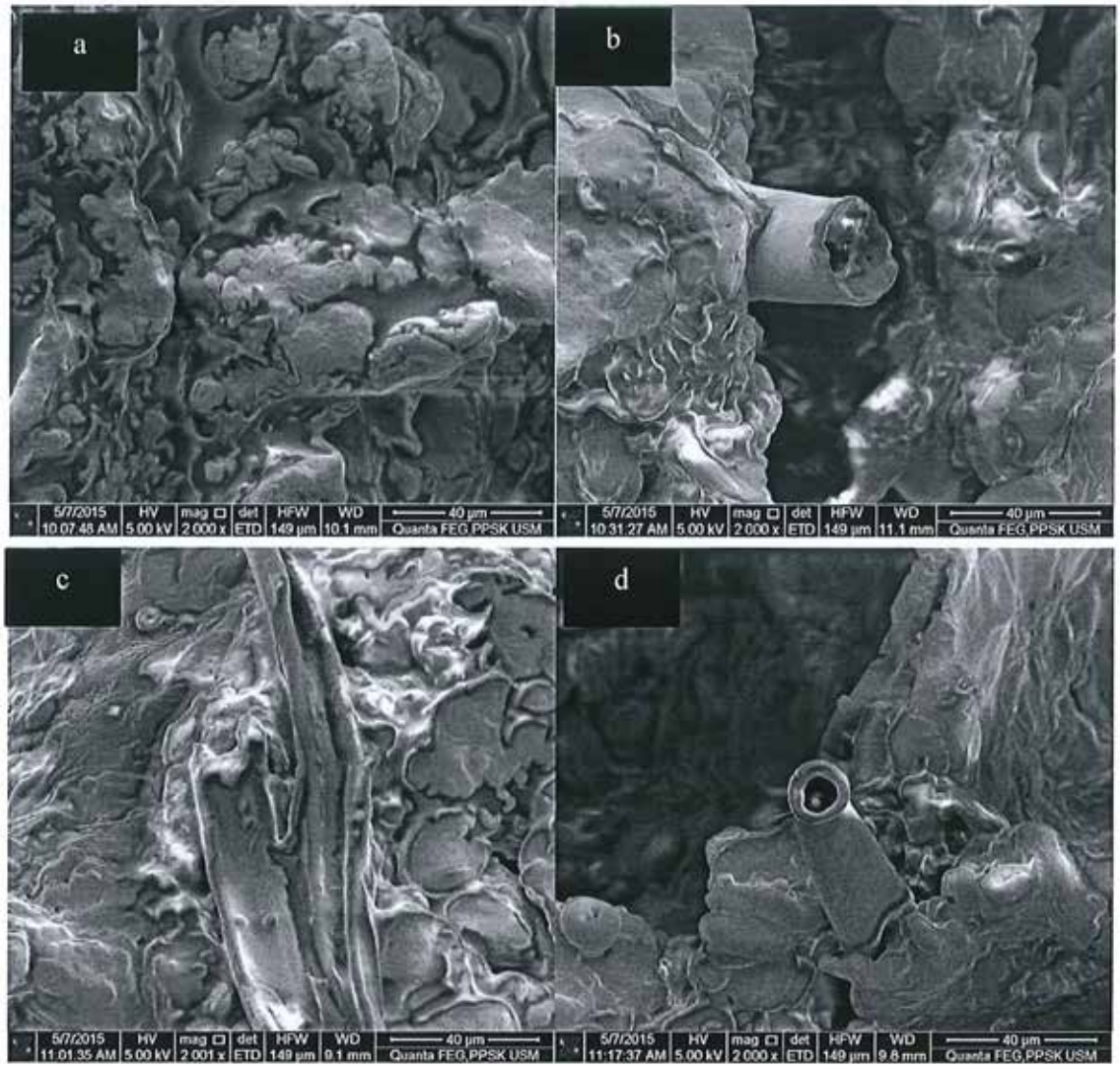

Figure 2: Scanning electron photomicrographs at higher magnification (2000X) of bun added without CLP and with CLP. 2a: is bun sample containing 0% of cornlettes (control), 2b: bun containing 4% with 45µm PS ofCLP, 2c: bun containing 4% with 125µm PS of CLP, 2d: bun sample containing 4% with 250µm PS of CLP. Some of the fiber thread is either partially chopped (2b) or partially broken (2c), but it functionality to absorb the oil/fat is still occurred (2d).

Table 1: Sensory acceptability of CLP bun for mulated with different PS of CLP

	Sensory Attribute					
Treatment	Overall	Aroma	Color	Softness	Elasticity	Flavour
Control	5.13±1.2[a]	4.20±15[a]	5.43±14b	4.43±17[a]	4.43±15[a]	5.00±12[a]
45 µm	4.57±15[a]	4.50±14[a]	4.80±1.4[ab]	4.50±17[a]	3.97±18[a]	4.23±16[a]
125 µm	4.67±15[a]	4.37±17[a]	4.33±12[a]	4.30±16[a]	4.30±16[a]	4.50±1S
250 µm	5.07±12[a]	4.47±14[a]	4.57±12[a]	5.33±15[b]	4.43±15[a]	4.83±16[a]

* The value is the mean of triplicates ± standard deviation.
[a-b]Mean values within the same column bearing different superscripts differ significantly (P<O.05)

CONCLUSION

There are irregular features of cornlettes fiber which sticked/attached to the cross-sectional surface of bun. Different PS of CLP formulated in bun revealed that they are minimally affected by the mechanical forces during preparation and baking process. Baking of proofed dough has been seen to minimally affect the structural integrity of cornlettes fibers which allow them to expand during bun-making. Some of the cornlettes fibers are prominently embedded in the gelatinized starch-gluten baked bun structure. Micro hollow structural feature of dietary fiber is able to absorb certain amount of fat molecules which are crucial in the development of soft and high quality baked-based product. 1n addition, all sensory attributes of bun formulated with CLP are not significantly different with the control bun. Addition of 250μm PS of CLP in bun has resulted in yielding softer and elastic bun. 1t could be concluded that CLP bun with the 250μm receive the most tolerable mean score of sensorial acceptability compared to other formulation and is closest to control bun.

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Evaluation of Antibacterial Effect of *Swietenia macrophylla* King Extract against

five bacteria species from Human Diabetic Wound Injuries Using Scanning Electron Microscopy

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ABSTRACT

This study has been designed to investigate the protective effects of *Swietenia macrophylla* King (seeds and endocarps) methanol extract, and to evaluate its antimicrobial activity against common pathogenic microbes isolated from human diabetic wounds using scanning electron microscopy (SEM). Serial swabs of 20 diabetic patients who were suffering of diabetic wound injuries we screened and there were 5 common bacterial species isolated, includes *E.coli, Klebsiella pneumoniae7, Pseudomonas mallei, Klebsiella aerogenes* and *Proteus sp.* The MIC and MBC methods were used to investigate the effectiveness of the plant extraction against these species. In addition, we evaluated the antimicrobial activity of the extract towards these microorganisms by observing the bacterial cell damage, for the treated and untreated microbes, using scanning electron microscope. The use of this extract, at certain concentrations, inhibits the growth of *Pseudomonas mallei, E.coli and Klebsiella pneumoniae* but however, *Klebsiella aerogenes* and *Proteus sp.* showed resistance to the extract. Observation using SEM reveals that the antimicrobial activity of the extract against tested bacteria demonstrated cell damage, elongation of cells and inhibitions of cell division. The results obtained also revealed the effectiveness of the extract against these microbes, as well as the antimicrobial potency to inhibit and damage bacterial cells which we believe that this extract could be used to develop a promising natural antimicrobial agent, that serves to avoid, treat, or lessen most of the complications of wound infections especially seen in diabetic patients.

Keyword: *Swietenia macrophylla* King, Antimicrobial effects, Scanning Electron Microscope, Diabetic Wound.

INTRODUCTION

Diabetes mellitus (DM) is a chronic disorder caused by inheritance or acquired deficiency, which develops either due to insufficient production of insulin by the pancreas, or ineffectiveness of the peripheral insulin hormone produced. It affects nearly 4% of the population worldwide, but this ratio is expected to increase by 5.4% in 2025 [1] and up to 7.7% (439 million adults) by 2030 [2]. DM is a multisystemic chronic disease that is frequently complicated by complex wound infections, due to the slowing down ability of the body to fight infections, because of high blood sugar which can lead to higher levels of glucose in the peripheral tissues, allowing bacteria to grow and infection to develop rapidly [3]. Medicinal plants are generally used for the treatment of various diseases, and many potent drugs have been purified from medicinal plants which range from anti-bacterial, anti-malarial, anti-diabetic and anti-cancer [4-5], moreover, diabetic wound infections have also being investigated [3]. The Ethno-pharmacological surveys indicated that more than 1200 plants are used worldwide in traditional medicine for their alleged antidiabetic activity, and these included Meliaceae family of Malaysian *Swietenia macrophylla* King [6]. *Swietenia macrophylla* King, commonly known as big leaf mahogany (vernacular) and 'skyfruit' (local), is a large tree from Malaysia, which reaching a height of 30–40 m and a girth of 3–4m, trunk of the tree is straight, cylindrical with a buttressed base. Flowering and fruiting are distinctly seasonal, the fruits may be produced once a year, and the trees start to produce their fruits regularly when about 15 years of age [7]. *S. macrophylla* King is used to treat diabetes and high blood

pressure in Malaysia [6-8]. Its seeds have been reported to have anti-inflammatory, anti-mutagenic and anti-tumor activities [9] and are also known to be effective against diabetes in rats [11]. In Chinese pharmacology and other traditional medicines, this plant has antipyretic, antifungal, and antihypertensive properties. Although most of the anti-diabetogenic studies (using animal models) have focused and confirmed the antidiabetic efficacy of seeds extracts [10-11], but there were still other recent studies which have proven the effectiveness of endocarps extract [13] as well for treatment of diabetes. Therefore, this study was designed to investigate the protective effects of *S. macrophylla* King seeds and endocarps ethanol extract, and to evaluate its antimicrobial activity against pathogenic bacteria isolated from human diabetic wounds using scanning electron microscopy.

MATERIALS AND METHODS

1.1 Patients samples

Serial swabs of 20 diabetic wound samples were obtained from each wound of the diabetic patients who underwent treatment at the local hospital in Kuala Lumpur Malaysia.

1.2 Bacteria Isolation and Identification

Samples were prepared using cultivation and streaking methods, and then microbial isolation and identification with biochemical tests and gram staining being executed. The bacteria were sub-cultured to isolate a pure growth onto nutrient agar for viabilities testing. Among 20 patients' samples, identifications of five common isolated bacterial species were obtained, these includes *Pseudomonas mallei*, *Klebsiella pneumoniae*, *Escherichia coli*, *Klebsiella aerogenes* and *Proteus sp*. Stock cultures were maintained on nutrient agar slant at 4°C and then sub-cultured in peptone water at 37°C prior to each antimicrobial test.

1.3 Plant Extract

S. macrophylla King's seeds together with its endocarps were collected from the northern part of Malaysia and were taxonomically identified. The selected plant parts were dried, crushed in an electric grinder and pulverized into a coarse powder form, out of this powder, 100g were weighed. The methanolic extraction was prepared by soaking 100g of the coarse powder in a conical flask with mixture solvent, consisting of 240ml distilled water and 320ml absolute methanol. The mixture was kept in an incubator at 37 ºC for 36 hours and stirred intermittently at 4 hours interval. It was then filtered, filtrated and dried under low pressure and low temperature rotary evaporator fitted with vacuum pump. A final 23.75g of the powder was collected at the end of the process. The samples were dissolved in normal saline at fixed dose for the treatment. Serial concentrations of the extraction were prepared in concentrations of 100%, 90%, 80%, 70%, 60%, and 50% repeatedly.

1.4 Antibacterial assay

Serial tubes of *S. macrophylla* King extract were mixed with peptone water up to achieve final concentrations of 100%, 90%, 70%, 60%, and 50%. The tubes prepared were then added each species of bacteria at 37°c.

For minimal bacterial concentration (MBC), bacterial cultures were inoculated into Mueller Hinton agar to determine the minimal concentration for inhibition.

1.5 Scanning Electron Microscopy

Bacterial species susceptible to the plant extraction were used for scanning electron microscope (SEM) observation. Cover slip was applied to the surface area of the inhibition zone of agar plate and left for 5 minutes. In addition, a 1 sq. cm of agar were cut and fixed in 3% (v/v) glutaraldehyde buffer solution in 0.1 M sodium phosphate buffer (pH 7.2) for 24 hours. The samples were then washed with sodium phosphate buffer for three times, followed by dehydration in a serial ascending concentrations of alcohol, 30%, 50%, 70%, 80%, 90%, 95% and 100%. The

specimens were then dried and mounted onto stubs and the samples then observed in Hitachi SU3500 Scanning Electron Microscope.

RESULTS AND DISCUSSION

1.1 Antibacterial assay of *S. macrophylla* Extract

The antimicrobial activity of *S. macrophylla* King methanol extract against the pathogenic bacteria were assessed by the presence or absence of inhibition zones and compared to positive and negative controls. Growth of *E.coli* was inhibited at 3 hours incubation at a concentration of 100% and 90%. However at a concentration of 70% and 80% inhibition of bacterial growth was only obtained after 24 and 48 hours incubation, respectively, *E.coli* incubated with extracts at a concentration of 50% and 60% showed absence of inhibition after 24 and 48 hours (Table 1).

Table 1: Antimicrobial effects of *Swietenia macrophylla* King extract against *E. coli*

E. coli								
Time	(+ve)	(-ve)	Extract Concentration					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	+	-	+	+	+	+	+	+
3 hours	+	-	-	-	+	+	+	+
5 hours	+	-	-	-	+	+	+	+
24 hours	+	-	-	-	-	-	+	+
48 hours	+	-	-	-	-	-	+	+

Klebsiella pneumoniae, showed inhibition of growth at 1 hour incubation at concentration of 90% and 100%, whereas, incubation at 80% of extracts concentration after 3 hours were inhibiting, but however, at the concentration of 50%, 60% and 70% there were no inhibitory zone. The results showed that all concentrations of the extract were able to inhibit *K. pneumonia* at 24 and 48 hours of incubation (Table 2).

Table 2: Antimicrobial effects of *Swietenia macrophylla* King extract against *K. pneumoniae*

Klebsiella pneumoniae								
Time	(+ve) Control	(-ve) Gentamycin	Extract Concentration					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	+	-	-	-	+	+	+	+
3 hours	+	-	-	-	-	+	+	+
5 hours	+	-	-	-	-	+	+	+
24 hours	+	-	-	-	-	-	-	-
48 hours	+	-	-	-	-	-	-	-

Pseudomonas mallei at 0, 1, and 3 hours incubation showed no inhibition for all concentrations. The inhibition zones were observed after 5 hours incubation, but however, the results showed that all concentrations of *S. macrophylla* extract were able to inhibit *P. mallei* after 24 to 48 hours of incubation (Table 3).

Table 3: Antimicrobial effects of *Swietenia macrophylla* King extract against *P. mallei*

Pseudomonas mallei								
Time	(+ve) Control	(-ve) Gentamycin	Extract Concentration					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	+	+	+	+	+	+	+	+
3 hours	+	+	+	+	+	+	+	+
5 hours	+	+	-	-	-	+	+	+
24 hours	+	-	-	-	-	-	-	-
48 hours	+	-	-	-	-	-	-	-

Klebsiella aerogenes and *Proteus sp.* showed no inhibition of growth at different time intervals of incubation for all concentrations, and thus, it suggests that this bacteria is resistant to the extract (Table 4. 5).

Table 4: Antimicrobial effects of *Swietenia macrophylla* King extract against *K. aerogenes*

Klebsiela aerogenes								
Time	(+ve) Control	(-ve) Gentamycin	Extract Concentration					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	+	-	+	+	+	+	+	+
3 hours	+	-	+	+	+	+	+	+
5 hours	+	-	+	+	+	+	+	+
24 hours	+	-	+	+	+	+	+	+
48 hours	+	-	+	+	+	+	+	+

Table 5: Antimicrobial effects of *Swietenia macrophylla* King extract against *Proteus sp.*

Proteus sp.								
Time	(+ve) Control	(-ve) Gentamycin	Extract concentration %					
			100%	90%	80%	70%	60%	50%
0 hours	+	+	+	+	+	+	+	+
1 hours	+	+	+	+	+	+	+	+
3 hours	+	+	+	+	+	+	+	+
5 hours	+	+	+	+	+	+	+	+
24 hours	+	+	+	+	+	+	+	+
48 hours	+	-	+	+	+	+	+	+

1.2 SEM Observation of Antibacterial Effects

To observe the morphological alterations of the effects of *S. macrophylla* King extract on bacterial species studied, samples prepared for scanning electron microscopy. Bacterial cells treated with extract were compared with untreated cells (control). Results showed that treated cells appeared shrunk and degradation of the cell wall was observed with noticeable damage to the outer layer of the cell wall. The extract was also able to inhibit the growth of the bacteria, as shown by decrease in amount and elongation of bacterial cells (Figure 1).

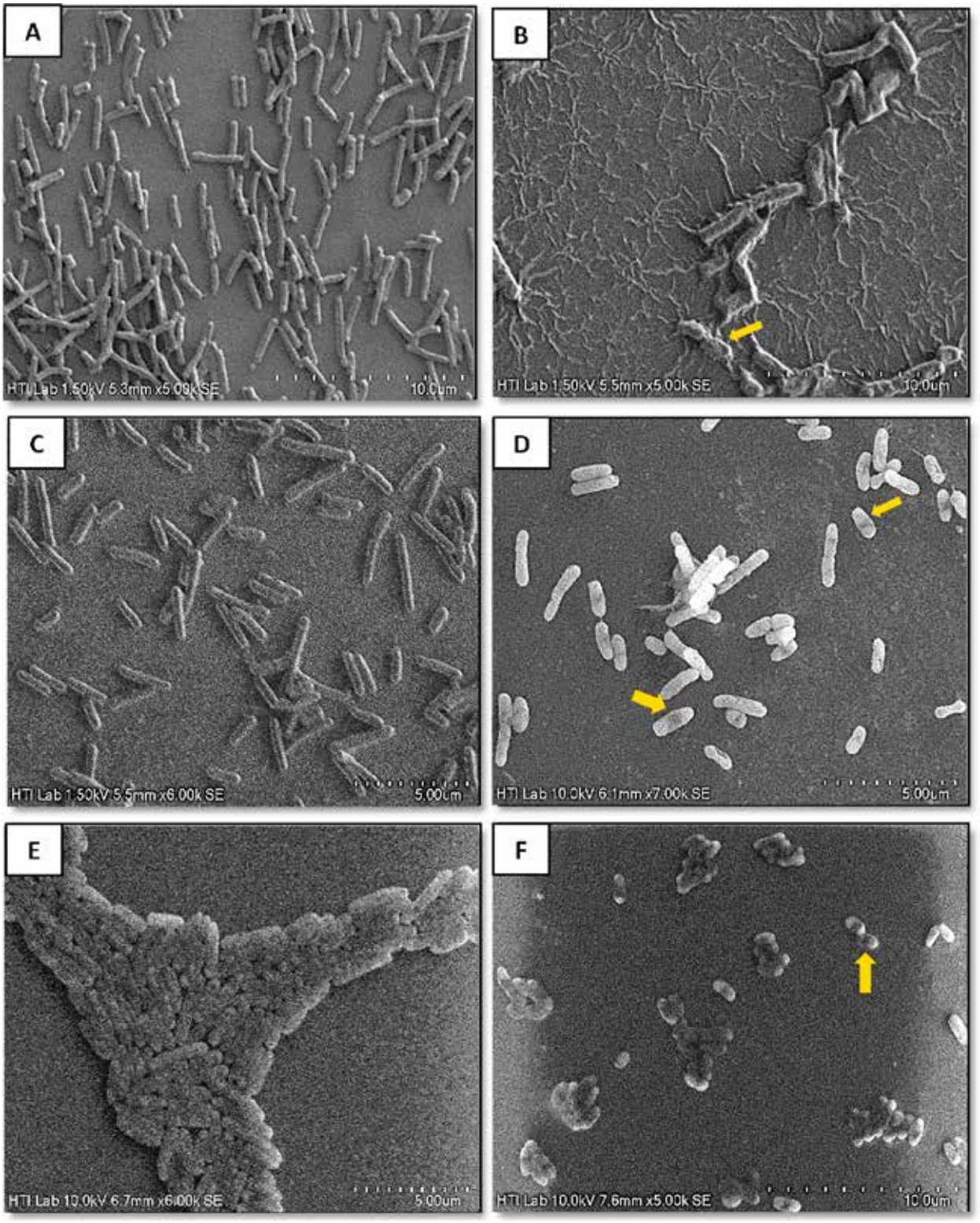

Figure 1: Comparison between untreated and treated bacteria with *S. macrophylla* King extract. (A) Morphology of untreated *E.coli*. (B) Morphological alterations of *E.coli* treated with extract. (C) Morphology of untreated *Klebsiella pneumoniae*. (D) Morphological alterations of *Kebsiella pneumoniae* treated with extract. (F) Morphology of untreated *Pseudomonas mallei*. (G) Morphological alterations of *Pseudomonas mallei* treated with extract. *The abnormalities of bacterial cells was as indicated by the arrow drawn.*

This study evaluated the protective effects of *S. macrophylla* King methanol (seeds and endocarps) extract against bacteria species isolated from human diabetic wound injuries. Previous test using diabetic animal models had proven its protective effect on the pancreatic islets of Langerhans histologically and morphologically, apart from that, hypoglycemic effects of *Swietenia* seeds [8-12], as well as, endocarps [13] had been confirmed previously. However, the antibacterial effects of methanol extraction of *S. macrophylla* King seeds and endocarps was only done in this present study. Diabetes mellitus was known to slow down the ability of the body to fight off infections due to the hyperglycemic state where high levels of glucose in the peripheral tissues favors and accelerate the growth bacteria and thus leading to infections that develops rapidly [4]. This study had revealed the antibacterial properties of *S. macropphylla* King against pathogenic bacteria determined using minimal bactericidal concentrations, which agreed with previous studies used different extractions of this plant and also reported its antibacterial activity, as well as, antifungal activity [5,9,10]. In the current study, the antibacterial potencies of this extract have been supported, as evidence, by SEM observations. The extract was effective to cause morphological alterations on the cell wall, and thus, revealed its antimicrobial mechanism by causing severe lysis and degradation of bacterial cell wall.

CONCLUSION

S. macrophylla King methanol extraction showed antimicrobial activities against five pathogenic bacterial species, by which, it causing degradation of cell wall and shrinkage of the bacterial cells.

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Histopathological Alterations in Organ Structures of Induced-Obese Rats Fed with High-Fat Diet (HFD)

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ABSTRACT

Obesity is excessive fat accumulation that increases the risk of cardiovascular disease, especially hypertension. World Health Organization (2013) reported approximately 2.8 million deaths per year due to obesity. This study is aimed to evaluate the vascular sensitivity and histopathological changes in the liver and aorta of diet-induced obese rats. Six male Sprague-Dawley (SD) rats, were fed with normal rat chow (n=3) and HFD (n=3) (cholesterol 32%) *ad libitum* for six weeks. Histological evaluation was carried out using hematoxylin-eosin (HE) stain and scanning electron microscopy (SEM). Femoral arterial rings were subjected to *in vitro* functional myograph study. The result suggests HFD for six weeks were successful in inducing the rats to become obese. Hematoxylin-eosin stain revealed a foamy degeneration of hepatocytes (acute fatty liver stage) in obese rats. SEM also showed a rough liver surface in obese rat. In aorta, no histological abnormality was observed for both groups. The maximum contractility in obese rats was significantly higher than normal rats suggesting hypersensitive response. This study suggests by using HFD to induce obesity in rats, the effects of obesity on health and diseases could be evaluated precisely.

Key words: obesity, HFD, myograph, histological, liver

INTRODUCTION

Obesity is an increasingly non-communicable disease problem in almost all developed countries including Malaysia, where 45.3% of the population is obese (WHO, 2013; Bernama, 2014). Approximately 2.8 million deaths occur per year due to obesity. Thus, it has been noted as the fifth risk factor for morbidity and mortality in Malaysia (WHO, 2014).

Obesity is defined by World Health Organization as an excessive fat accumulation (WHO, 2014), which can increases the risk of the development of various diseases including: insulin-resistant diabetes mellitus, endocrine problems, certain forms of cancer, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (Dobrian *et al.*, 2000; Williams *et al.*, 2002; Landsberg *et al.*, 2013).

Non-alcoholic fatty liver disease is identified as lipid accumulation in individuals who consume less than 20g ethanol/day. The hallmark feature of NAFLD is steatosis; an infiltration of liver cells with fat. With increasing body mass index (BMI) the prevalence rate of NAFLD also increases. Fabbrini *et al.* (2010) stated the prevalence rates of steatosis are approximately 15% in non-obese persons. In persons with class I and II obesity (BMI 30.0-39.9 kg/m2) the prevalence are 65% and in extremely obese patients (BMI 40 kg/m2) are the highest (85%).

Cardiovascular disease mainly hypertension has been shown by numerous study to be associated with obesity (Hadi *et al.*, 2005; Kotsis *et al.*, 2010; Lobato *et al.*, 2012), whereby hypertension is associated with vascular changes and damage (Olsen *et al.*, 2004; Virdis, 2011). Among the features of vascular damage is endothelial dysfunction. In endothelial dysfunction, there is an increased in vascular response upon stimuli, which predispose to development of hypertension (Deanfield *et al.*, 2007; Kotsis *et al.*, 2010).

Although obesity is strongly associated with hypertension and NAFLD, the effectiveness of self-prepared HFD to correlate obesity and both diseases remains to be elucidated. Therefore, the present study aimed to investigate the changes of body weight and BMI of Sprague Dawley

rats after feeding self-prepared HFD for 6 weeks. At the same time, co-morbidities risks related to obesity such as blood pressure and function of blood vessel were also measured. Histology of liver and section of the aorta were also observed in order to assess any alteration of the structure.

MATERIALS & METHODS

Animals and Drugs Used

6 male Sprague-Dawley (SD) rats (3 months old; 200-250g) were used in this study for pathophysiological examinations and physiological vessel contraction experiments. The animals used for the experiments were obtained from Animal Research and Service Centre of Universiti Sains Malaysia (ARASC). They were housed individually in the animal room controlled at 22 ± 1 °C and a light: dark-cycle of 12h: 12h. Experiments were performed after the approval by Animal Ethics Committee USM (USM/Animal Ethics Approval/ 2015/ (95) (635)).

SD rats were equally divided into two groups; normal control (n=3) and obese group (n=3). For control, each animal was fed standard rat chow (Gold Coin Feedmills (M) Sdn Bhd, Malaysia) *ad libitum*. Obese group was fed self-prepared HFD (32% fat) *ad libitum* for 6 weeks to induce obesity. All rats were allowed free access to water.

At the end of 6 weeks, rats were euthanized with an intraperitoneal overdose of sodium pentobarbitone (100 mg/kg), liver and thoracic aorta were isolated. Sodium pentobarbitone (Alfasan Woerden-Holland) and phenylephrine (Sigma-Aldrich, USA) were used.

Induction of Obesity

The rats were fed with HFD for 6 weeks to develop obesity. HFD used for this study was self-prepared by our own group. The HFD was prepare and modified according to Nik Norliza *et al.* (2014). The HFD consisting of 32g of ghee (saturated fat from animal), 300mg calcium and 100 IU vitamin D3 added to 68g of powdered standard rat chow and thoroughly mixed. The mixture was then made in a dough-like consistency and presented as marble shape. The HFD was kept in refrigerator at 4°C to make it firm and hold the shaped before giving to the respective group.

Anthropometrical Determination

Body weights and body length (nose to anus length) were measured weekly. The body weight and body length were used to determine BMI (Novelli *et al.*, 2007):

Body mass index (BMI) = body weight (g)/length2 (cm^2).

Histological Examination

Hematoxylin and Eosin Staining

Each tissue was fixed 3 days in 10% formaldehyde at room temperature. Tissues were embedded in paraffin and cut into 4 μm cross-sections. Microscopic examination was performed with hematoxylin-eosin stained sections to assess fatty changes in liver, and in aorta.

Scanning Electron Microscope (SEM)

Each tissue was fixed 24 hours in McDowel-Trump Fixative at 4°C. Tissues were then cut, washed, post-fixed and dehydrated. Next, tissues were processed with critical point drying step, coated with gold. Both tissues were then viewed under Quanta FEG 450 Scanning Electron Microscope (SEM) by using XTm Product Version 4.1.7.2095 viewer software.

Measurement of Vascular Contracting Function

Femoral arteries were isolated and excess fat and connective tissues were removed. Vessels were cut into rings 1 – 1.5 mm long. Artery ring preparations were suspended and incubated in organ baths containing modified Krebs-Henseleit solution gassed with 95% O2 and 5% CO2 (37 °C, pH 7.4). The solution contained 118.4 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.1 mM glucose and 2.5 mM $CaCl_2$.

Changes in isometric force were recorded on a computer by use of Chart program (version 6.0) and a PowerLab data acquisition system (ADInstruments Ltd, Oxfordshire, UK). The resting tension was set at 1.0 g (Shi *et al.*, 2007). After an equilibration period of 30 min, each preparation was contracted with 40 mM potassium chloride (KCl) repeatedly to assess the viability of the artery. The solution was changed 3 times after the contraction reached plateau. Only rings which give contractile response of more than 50% of maximum contraction were chosen.

Then, artery preparations were then contracted with phenylephrine in cumulative dose; 1nM, 3nM, 10nM, 30nM, 100nM, 300nM, 1μM, 3μM, 10μM, 30μM, 100μM, 300μM and 1mM accordingly to measure the contraction response (Figure 1). The cumulative dose response curves for phenylephrine contractions were fitted with non-linear method using GraphPad PRISM version 6.0.1 for Windows. The effective concentration that causes 50 % of maximal effect (EC_{50} values) was then obtained from the curve.

Statistical Analysis

All values used in analysis are presented as means ± SEM. Comparisons among the different groups were performed by unpaired t-test using GraphPad PRISM version 6.0.1 for Windows (GraphPad Software, San Diego California USA). All test were considered significant when $p < 0.05$.

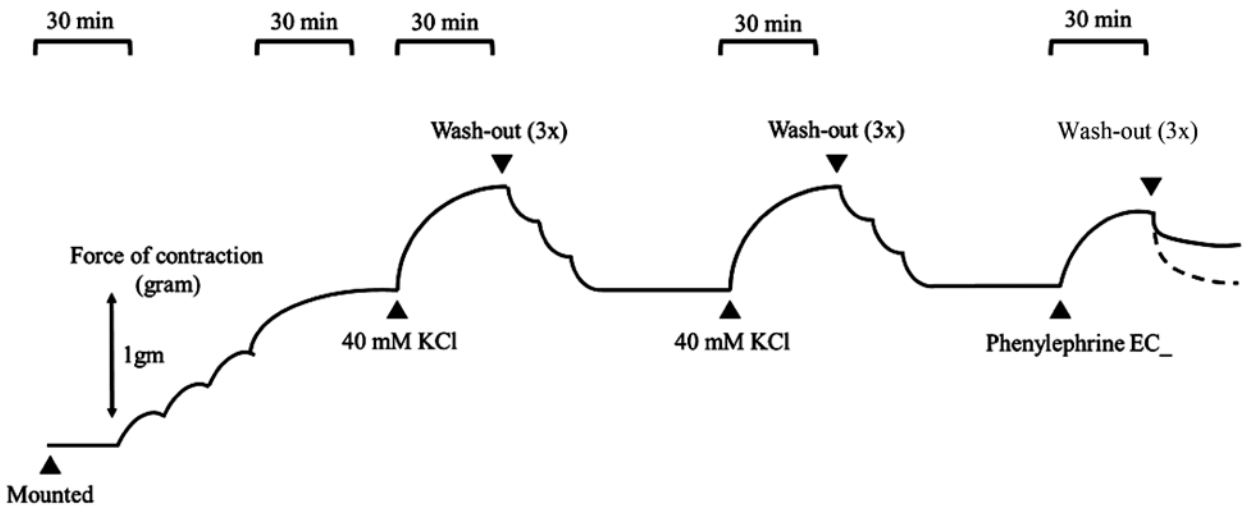

Figure 1. The protocol to measure the response of vascular contraction by phenylephrine in femoral artery. Each preparation was pre-contracted with KCl (40 mM) to assess the viability.

RESULTS

Effect of HFD on Body Weight, Body Mass Index (BMI) and Percentage of Body Weight Gain.

Similar to humans, evaluation of obesity in rats also can be assessed and confirmed by comparing (1) body weight, (2) body mass index and (3) percentage of body weight gain between normal group and induction groups of the animals. Table 1 summaries the comparison changes of body weight, body length, BMI and percentage (%) of body weight gain of the rats after induction for 6 weeks. Based on the results, the mean value of body weight, BMI and % body gain of both groups over the study of 6 weeks increased with the period of induction.

Statistically, there were significant elevation (p=0.021 and p=0.017 respectively) in the final weight measurements (g) and BMI (g/cm^2) in obese rats, when compared to normal rats (Figure 2.1 and Figure 2.2). Apart from that, the result also demonstrated that there was a significant increase

Table 1: A Distribution of Weekly Mean Body Weight, Body Length, Body Mass Index (BMI) and Percentage of Body Weight Gain of Normal and Induced-High Fat Diet for 6 Weeks in SD Rats (Confirmation Study)

Weeks	Mean Body Weight ± SEM (g)		Mean Body Length ± SEM (cm)		Mean BMI ± SEM (g/cm^2)		Mean Percentage of Body Weight Gain ± SEM (%)	
	Normal rats (n=3)	**Induced rats** (n=3)	**Normal rats** (n=3)	**Induced rats** (n=3)	**Normal rats** (n=3)	**Induced rats** (n=3)	**Normal rats** (n=3)	**Induced rats** (n=3)
0	225.7 ± 2.4	231.7 ± 1.5	22.8 ± 0.07	22.7 ± 0.12	0.43 ± 0.01	0.45 ± 0.01	-	-
1	308.7 ± 8.6	380.0 ± 8.5	22.8 ± 0.07	22.7 ± 0.12	0.59 ± 0.02	0.73 ± 0.02	36.8 ± 3.2	64.0 ± 3.6
2	333.7 ± 6.3	432.3 ± 2.6	22.8 ± 0.07	22.7 ± 0.12	0.64 ± 0.01	0.84 ± 0.01	47.9 ± 3.1	86.6 ± 1.1
3	338.0 ± 5.3	450.3 ± 4.4	22.8 ± 0.07	22.7 ± 0.12	0.65 ± 0.01	0.87 ± 0.02	49.8 ± 2.9	94.4 ± 1.3
4	340.3 ± 5.5	474.7 ± 4.2	22.9 ± 0.03	22.8 ± 0.13	0.65 ± 0.01	0.92 ± 0.01	50.8 ± 2.8	106.3 ± 1.0
5	344.3 ± 5.4	497.0 ± 8.5	22.9 ± 0.06	22.8 ± 0.10	0.65 ± 0.01	0.95 ± 0.02	52.6 ± 2.6	114.6 ± 3.9
6	346.7 ± 4.8	522.7 ± 5.7[a]	22.9 ± 0.06	22.8 ± 0.12	0.66 ± 0.01	1.00 ± 0.01[a]	53.7 ± 2.6	125.6 ± 2.8[c]

Note: **a**: *p<0.05* when compared to Normal rats; **c**: *p<0.001* when compared to Normal rats.

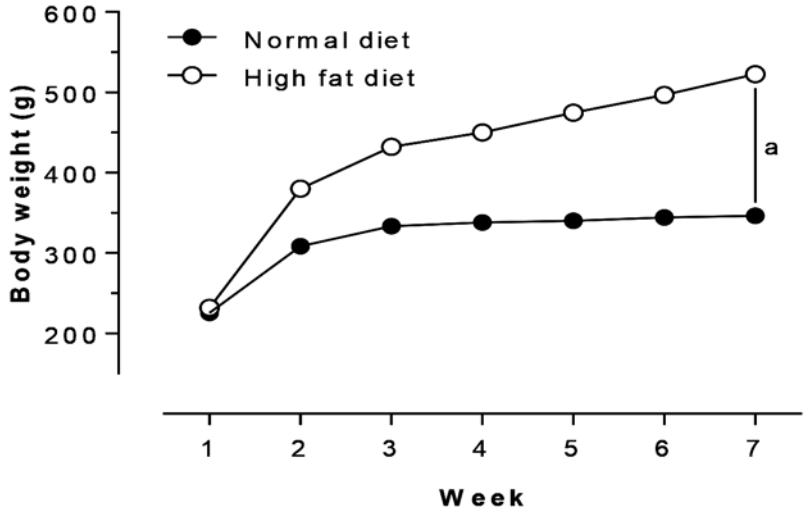

Figure 2.1: Body weight in response to high fat diet and normal rat chow on over period of 6 weeks in Confirmation Study. a: $p<0.05$, using unpaired-t test (n=3).

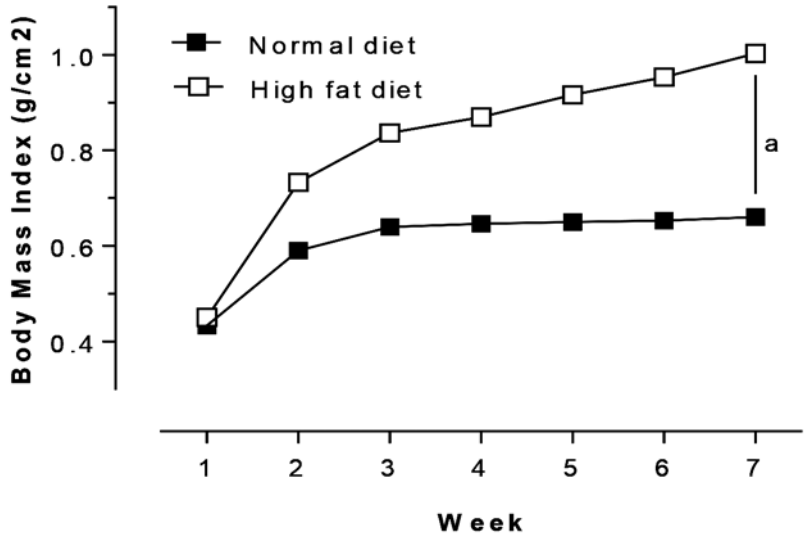

Figure 2.2: Body mass index (BMI) in response to high fat diet and normal rat chow over period of 6 weeks in Confirmation Study. a: $p<0.05$, using unpaired t-test (n=3).

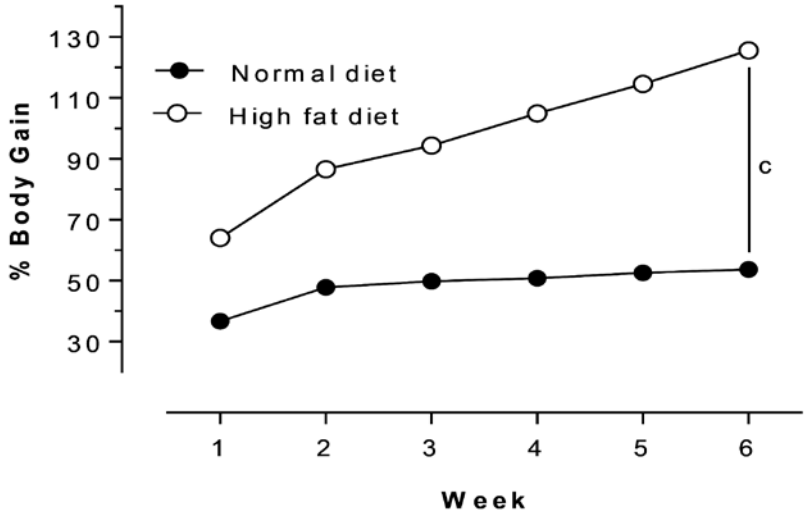

Figure 2.3: Body weight gain in response to high fat diet versus normal rat chow over period of 6 weeks in Confirmation Study. c: $p<0.001$, using unpaired t-test (n=3).

of percentage body weight gain on obese rat (p=0.003) in comparison to normal rat (Figure 2.3). This result indicates that percentage of weight gain was double up in obese rats compared to normal rats.

Histological Examination of the Liver and Aorta

In the normal rats, HE staining of liver did not show any pathological changes (Figure 3a). In contrast, accumulation of fat in the liver increased in obese rats (Figure 3b). Hepatocytes were swelled and enlarged. The fatty changes were remarkable throughout the tissue. Some liver cells showed the foamy degeneration of hepatocytes. These foamed hepatocytes were several times larger than normal liver cells indicating steatosis (acute fatty liver stage).

In descending thoracic aorta portion, no histological abnormalities were observed in normal rats (Figure 3c). While obese rats showed slightly thickening of vessel layer (Figure 3d).

For SEM study, liver of obese rat showed a rough surface (Figure 4b) and formation of fat accumulated on the surface (Figure 4c) as compared to normal rat; which has smooth hepatic lobular architecture (Figure 4a).

Vascular Contraction Response of Femoral Artery

Functional myograph study shows that arterial ring maximal contraction was significantly higher in obesed rat compared to normal rat ($p<0.05$ real value?). The EC_{50} of obese rats was 3.1 μM with the Emax of 5.12 + 0.17 gm whereas for normal rat, the EC_{50} is 3.4 μM with the Emax of 2.98 + 0.16 gm (Figure 5).

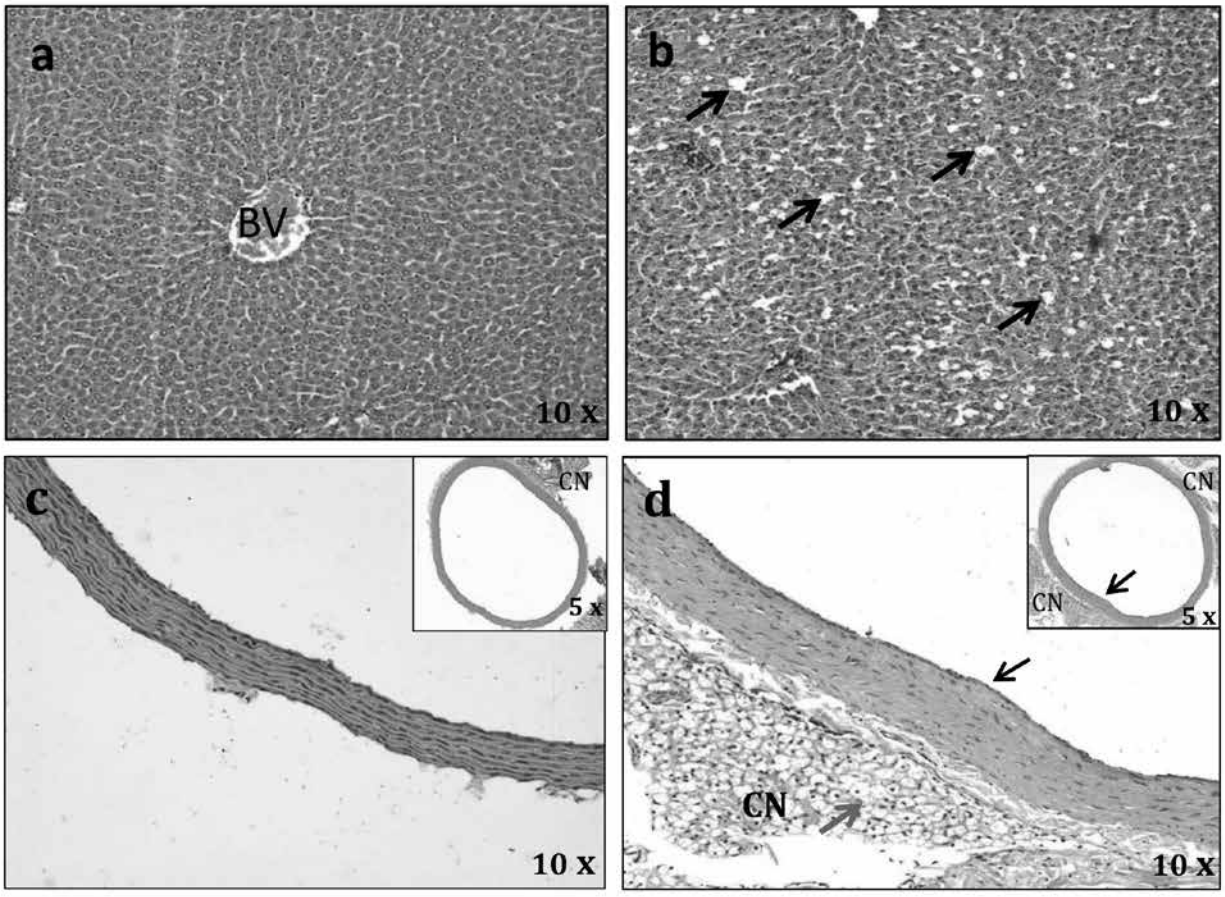

Figure 3: The liver and aorta histology in response to HFD under light microscope using Hematoxylin & Eosin (HE) stain with magnification 10× and 5× respectively. (a) normal liver lobule; (b) structural alteration and fat accumulation (black arrow) in liver induced rats; (c) normal aorta section; (d) descending thoracic aorta of induced rats, with slightly intimal thickening (black arrow) and increase of fat deposit in connective tissue (blue arrow) in (BV = blood vessel; CN = connective tissue.

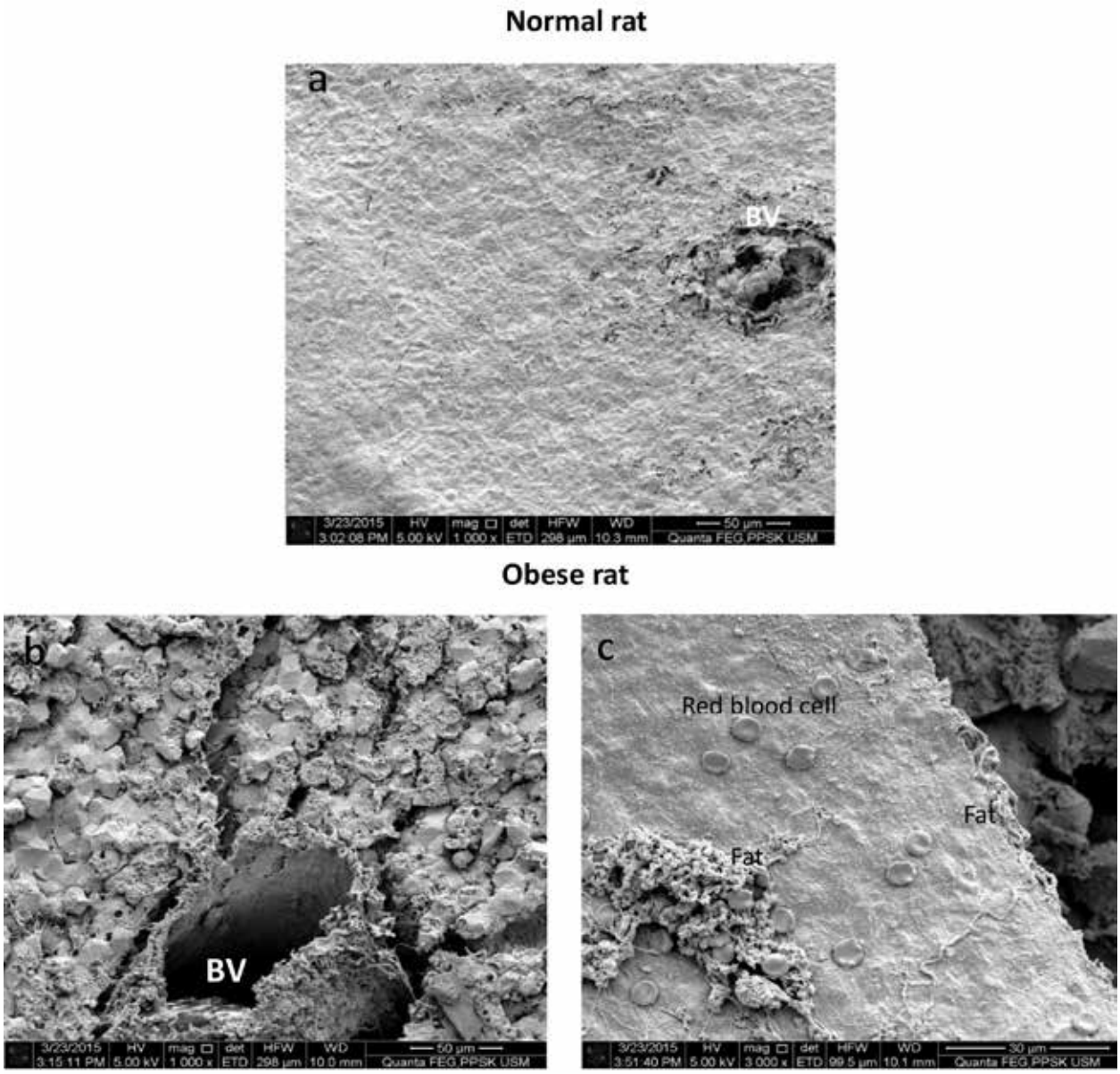

Figure 4: Scanning Electron Microscope images showing the effects of treatment diets in liver tissue under (high vacuum). (a) normal liver surface of normal rat; (b) rough liver surface of obese rat; (c) fat accumulation on the liver surface of obese rat. (BV = Blood vessel)

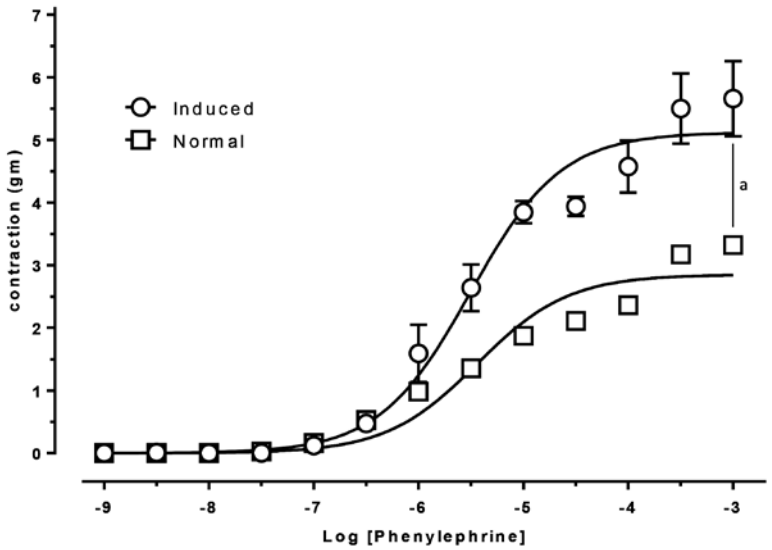

Figure 5: Contractile response of femoral arterial ring to phenylephrine, as contractile agent. The arterial of obese rat is significantly hypersensitive to phenylephrine. a: $p<0.05$, using unpaired t-test (n=3).

DISCUSSION

Several studies have suggested that obesity determination in animals has been confirmed to be similar as human obesity determination by comparing (1) body weight, (2) body mass index and (3) percentage of body weight gain between normal group and induction group(Woods *et al.*, 2003; Buettner *et al.*, 2007; Mamikutty *et al.*, 2014)**.** In accordance to Novelli *et al.* (2007) normal BMI of male rat was documented in the range of 0.45 ± 0.02 g/cm² to 0.68 ± 0.05 g/cm². Therefore, BMI value exceeds 0.68 g/cm^2 is considered as obese.

Consistent with several studies, free access to HFD for 6 weeks used in this study was effective and successful in promoting a significant increase of body weight and BMI in the rats, thus verifying the obese status; as all induced rats showed BMI values above 0.68 g/cm^2 (Warwick *et al.*, 1992; Buettner *et al.*, 2007; Gajda, 2009; Neyrinck *et al.*, 2009). Therefore, feeding HFD has been evaluated as the most reliable tool to create obese models as the rats prone to gain weight in a quick time (Gajda, 2009) than those on diets containing low amounts of fat (Deuel *et al.*, 1944; Deuel *et al.*, 1947). Moreover, its high similarity in mimicking the common route of human exposure (Buettner *et al.*, 2007) also became the appropriate tool in promoting development of obesity at nominal cost.

Other than that, the histopathological studies on the liver section were also performed. Liver was selected for histology study because this organ is responsible in fat metabolism (Nguyen *et al.*, 2008). Several studies have suggested induced obese with HFD commonly associated with inflammation, congestion and non-alcoholic fatty liver disease (NAFLD) which can lead to hepatic failure (Altunkaynak *et al.*, 2009; Kameshwaran *et al.*, 2013). Evidently, our histological examination demonstrated that consumption of self-prepared HFD for development of obesity also able to cause hepatocellular damaged within 6 weeks. The results showed that foamy degeneration of hepatocytes and structural changes depicted in liver section of obese rats when viewed under light microscope (H&E stain); suggestive steatosis. Whereas SEM image showing a rough liver surface of obese rats. Hence, from both examinations, we speculated that induced rats with self-prepared HFD might play a crucial role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD).

It is known that the pathogenesis of NAFLD is complicated. The suggested mechanism underlying the fatty liver is the “two-hit hypothesis”. The first hit is fat accumulation in the liver, which impairs fatty acids metabolism by its own. The second hit is probably due to pro-oxidative and hepatotoxic events (Day *et al.*, 1998). In addition, increased saturated fatty acid intake (SFA) is usually associated with high insulin resistance which can provoke NAFLD progression. Evidence shows that SFA accumulation in the liver can have detrimental effects on the liver function (Kani *et al.*, 2014).

Human studies have revealed that increased fat consumption in daily meal is also associated with other related metabolic diseases (Bais *et al.*, 2014). In this regards, several studies proved there were elevation of blood pressure in a diet-induced obese-rat model (Dobrian *et al.*, 2001; Nagae *et al.*, 2009; Spradley *et al.*, 2013). As expected, hypertension developed in obese rats whereby a significant result had been observed when compared the vascular contraction to normal rat.

Several mechanisms have been identified on the pathogenesis of obesity- induced hypertension. Of great interest, we proposed damage of endothelial as the major factor, owing to the fact that obesity represents a state of inflammation which can cause endothelial dysfunction (Fonseca-Alaniz *et al.*, 2007; Kotsis *et al.*, 2010). Endothelial dysfunction is characterized by an altered vascular function in which it would reduce the release of endothelium-derived relaxing factors, particularly nitric oxide (NO) and an increase in endothelium-derived contracting factors (Hadi *et al.*, 2005; Villar *et al.*, 2006) (Fernández-Sánchez *et al.*, 2011). These conditions would then promote the pressure changes and flow patterns which can result into obesity-associated hypertension (Hadi *et al.*, 2005; Villar *et al.*, 2006). Even in normotensive subjects, endothelial

function progressively deteriorates as blood pressure rises (de Jongh *et al.*, 2004).

Consistent to this evidence, a significant increase of vascular contractility has been observed when the vessel was exposed to contractile factors; phenylephrine. This indicates that vessel of induced-rat was hypersensitive to phenylephrine as compared to normal rats; suggesting endothelial damaged in induced-rats. The findings is consistent with study by Deanfield *et al.* (2007), in which phenylephrine caused an intact vasoconstriction in normal endothelium, but caused abnormal vasoconstriction in subjects with endothelial dysfunction, as a result of vasoconstrictor effect.

In addition, the study also revealed damaged of endothelial in obese is also characterized by its vessel structural changes (McIntyre *et al.*, 1999; Lobato *et al.*, 2012). To supplement the result, histology studies on aorta section was carried out. The result demonstrated that there was slightly thickening of vessel layer and fat deposition in connective tissue observed in obese rat. Studied by Kotsis *et al.* (2010) and Virdis (2011), stated that vascular changes might be mediated to cause endothelial dysfunction which predispose to hypertension.

CONCLUSION

This study showed that the marked elevation of body weight, fatty degeneration of liver and rapid onset of vascular dysfunction in endothelial cells in obese rats. These results suggest that self-prepared HFD is able to promote obesity in SD rats.

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Effect of Methotrexate on Stillbirth, Weight of Mice Embryos and Histopathological Changes of Embryonic Liver

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ABSTRACT

Methotrexate (MTX) is a folic acid antagonist, which is widely used as a cytotoxic chemotherapeutic agent for malignancies as well as for the treatment of psoriasis, rheumatic diseases and cancer. On another hand, it might cause serious or life-threatening toxicities on liver, lungs, kidney, and immune system. The present study was conducted to assess the harmful effect of the low dose of MTX on the stillbirth, weight of the mice embryos, and to determine the histopathological changes of the embryonic liver during two gestational ages. Thirty mice were divided into to three groups, of 10 pregnant mice each. The first group (G1) was injected low dose of MTX (0.02 mg/kg), at days 4, 5 and, 6 of gestation. The second group (G2) injected by same dose of MTX at days 14, 15 and 16. The third group (G3) served a control group and injected with 0.5 ml of normal saline. Pregnant rats sacrificed on gestational day 7 and 17 of gestation. Embryo weight and incidence of intrauterine death were recorded. Twenty mice embryos were fixed in the Bouin fixative and processed for histological study. The result showed a significant ($P< 0.05$) decrease in embryos' weight at days 7 and a highly significant reduction ($P< 0.01$) at days17 of gestation compared with control groups. In addition, there was a highly significant increase ($P< 0.01$) in mortality rate that was observed in the embryos of MTX treated pregnant mice. The histological examination showed fatty changes, degeneration, variability in nuclear size and staining, and necrosis of hepatocytes. These results confirm that administration of low dose MTX in early pregnancy has teratogenicity effect on liver development.

Keyword: Embryo, intrauterine death, liver toxicity, methotrexate, weight.

INTRODUCTION

Methotrexate (MTX) is an immunosuppressive and antifolate drug which is generally used at a low-dose to treat psoriasis, rheumatic diseases (Cronstein, 2005) and cancer (Colleoni *et al*., 2002). Stovall *et al* (1991), introduced single-low dose of MTX protocol for the treatment of extrauterine pregnancy at first-trimester terminations, and gestational trophoblastic disease.

Methotrexate binds to the enzyme dihydrofolate reductase (DHFR); thus inhibits thymidylate, serine and methionine synthesis, which disrupts synthesis of DNA, RNA, and protein leading to cell death (French and Koren, 2003). Other studies have shown that MTX inhibit DHFR, an enzyme reducing folic acid to tetrahydrofolic acid (Grossman *et al*., 2004). Tetrahydrofolates are utilized as carriers of one carbon fragments necessary for synthesis of purine nucleotides and thymidylate. Folic acid is needed for the *de novo* synthesis of the nucleoside thymidine, which is required for DNA synthesis. Also, folate is needed for purine base synthesis, so all purine synthesis will be inhibited. MTX, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins (Padmanabhan *et al*., 1986).

Methotrexate might reduce the intracellular glutathione concentrations leading to diminish the macrophage and lymphocyte recruitment and function (Cronstein, 2005). The long term therapy of methotrexate results to distribute it is concentration in the body tissues, extracellular fluids, and the highly concentrated was in the kidney, gallbladder, spleen, liver and skin (Lloyd *et al*., 1999). In humans, the MTX has been associated with fetal malformations in central nervous system

abnormalities, mental retardation, skeletal abnormalities, partial or absent ossification of bones, micrognathia, cleft lip or palate, broad depressed nasal bone, hypertelorism, short limbs, syndactyly, absent digits, and clubfoot and dextrocardia, and intrauterine growth retardation(Feldkamp and Carey, 1993; Lloyd *et al*., 1999).

With the long-term use of MTX as chemotherapy in high doses, or following chronic administration in rheumatic diseases, cancers and liver damage that involves fibrosis and even cirrhosis are the most important major side effect (Uraz *et al*., 2008). The hepatotoxicity of MTX has been related to the production of reactive oxygen species (ROS) (Richard *et al*., 2000; Uraz *et al*., 2008). The oxidative tissue damage of the MTX was associated with increasing lipid peroxidation and decreasing level of antioxidant enzymes in the liver (Vardi *et al*., 2010). Jordan *et al*, (1977) studied the teratogenicity of MTX on animals' embryos and observed cleft palate, skull defects, and severe fore- and hind limb dysplasia, these defects were strongly-dose dependent during the developmental stage of the embryo.

Although, MTX was originally designed as a chemotherapy drug in high doses, but low doses of the MTX ares well known to cause elevations in serum aminotransferase levels (ALT) and long-term therapy has been linked to development of fatty liver disease, fibrosis and even cirrhosis. There are variable rates of abnormalities in liver function test and in histopathological observations of liver biopsies at different doses. The trials with MTX showed that the drug has been well tolerated, although toxicities were encountered. Gastrointestinal toxicity, stomatitis, alopecia, marrow suppression, and liver function abnormalities were commonly encountered, although folic acid or folinic acid supplementation has diminished. The frequency with which low- dose MTX causes clinically significant liver fibrosis has been debated but does not appear to be a great risk (Richard *et al*., 2000).

The teratogenicity of MTX intoxication on the liver has been demonstrated in many experimental animals. Most of these studies used high-dose of MTX (20 mg/kg, I.P.) to pregnant rats at different days of pregnancy (Vardi *et al*., 2010). Hadi *et al*, (2012) gave MTX (200 mg/kg/day) orally to their adult rabbits for eight weeks, Baskerville and Cox (1978) treated adult mice with nine intraperitoneal injections of (1mg MTX /mouse) for 3 weeks. All these studies demonstrated degeneration of liver cells and irregularity in nuclear size. Therefore, the aims of this study were to assess the harmful effect of low dose of MTX (injected during gestation) on weight, stillbirth (intrauterine death) of mice embryos and to determine the histopathological changes of the mice liver during two gestational ages.

MATERIALS AND METHODS

Thirty female Swiss-Webster mice weighing 28-30gm and aged 6-8 weeks were kept under suitable environmental conditions such as a room temperature that was maintained at about (24+2^{o}C) and exposed to 12 hours/day light program. The animals were randomly divided into three groups with 10 animals each that involved two experimental groups (G1, G2) and control group (G3). Vaginal smears of all animals were performed to determine the regularity of at least three consecutive estrus cycles. Then, the animals that are in estrus phase were allowed to mate with mature healthy males. The occurrence of vaginal plug examination was performed to observe the spermatozoa and to determine the gestation period, which considered as the first day of pregnancy (Fakhrildin, 2000).

The experimental pregnant female mice group (G1) were injected IM with 0.5ml (0.02 mg/kg) MTX, at days 4, 5 and 6 of gestation, while the animals in the G2 group were injected by the same dose of MTX at days 14, 15 and 16 of gestation. The control group (G3) was injected with 0.5 ml of normal saline.

Ten pregnant mice of G1 and five pregnant female mice of G3 were sacrificed on the 7th gestational day and ten pregnant mice of G2 and five pregnant female mice of G3 were sacrificed on day 17 of gestation. The abdominal cavity was surgically opened and an incision was made in

the uterus and the umbilical cord connected to the fetus was cut.

The total number of implantations was 111 embryos that involved 76 embryos in treated groups and 35 embryos in control group. The number of stillbirth (dead) and live fetuses in each uterus horn were recorded. Each fetus for all groups was washed and weighed. For histological study, livers from 30 treated embryos (ten from each group) were fixed in Bouins fluid for 24 hours, dehydrated by 70% ethanol, and processed for light microscopy; the sections were stained by Harris hematoxylin and eosin.

The experimental animal protocol was conducted in compliance with humane animal care standards outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental study protocol was approved by Al-Nahrain University ethical committee NU, 112/ 2010.

RESULTS

The present study showed that the injection of MTX to pregnant mice caused a significant (*P< 0.05)* reduction in embryos' weight at day 7 as compared to the control group (Figure.1. A), whereas a highly significant reduction (*P< 0.01*) in the weight at day 17 of gestation was noticed as compared with the control group (Figure.1. B). None of the embryos died in the control group while there was a highly significant increase (*P< 0.01*) in mortality rate at day 7 and a significant increase (*P< 0.05)* in mortality rate at day 17 as compared with the control group (Figure.2).

Light microscopic examination of the embryos liver sections of the control group animals sacrificed at the 7th day of embryonic development showed normal architecture of hepatic tissue with developed hepatoblasts and portal areas (Figure. 3. A). On the 17th day of gestation, the liver showed normal architecture and completed hepatic lobulation of hepatic tissue with polygonal hepatocytes having regular nucleus and cytoplasm (Figure. 3. B).

Liver specimens collected from animals on 7th day after being treated with (0.02 mg/kg) MTX at 4, 5 and 6 days of gestation, showed severe changes. The major histopathological changes were in the total loss of hepatic architecture including disarrangement of hepatic strands. Highly marked degeneration and necrosis of hepatocytes was noted with loss of nuclei, vacuolization and apoptotic changes. Other hepatocytes appeared with a pyknotic nucleus and acidophilic cytoplasm as show in (Figure. 4. A-C).

Liver sections from mice injected with MTX at days 14, 15 and 16 and sacrificed on the 17th day of gestation showed hepatic fatty changes, variability in nuclear size and staining, which ranged from minimal to pyknotic nuclei, marked pleomorphism, giant cells and hepatocytes with vacuolations and hydropic degeneration. There was damaging of sinusoid and portal areas, and extracellular cells fat droplets accumulation (Figure. 5. A and B).

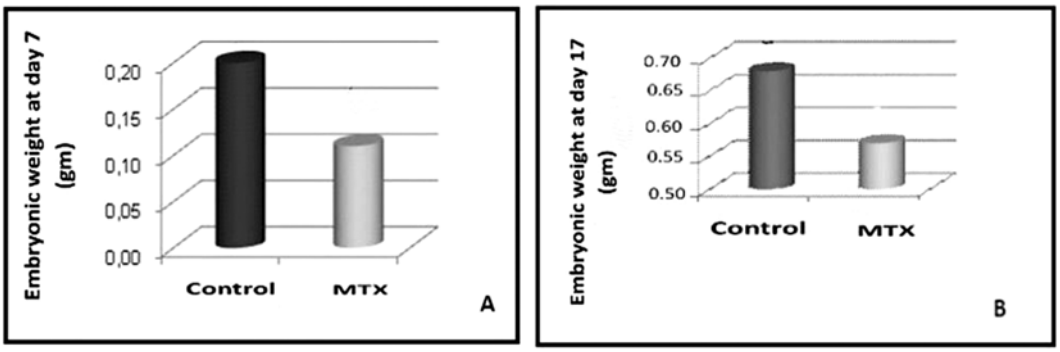


Figure- 1: Diagrams showing the effect of μm 0.02 (MTX) on the embryonic weight during the 7th (P< 0.05) (A) and the 17th (P< 0.01) (B) days.

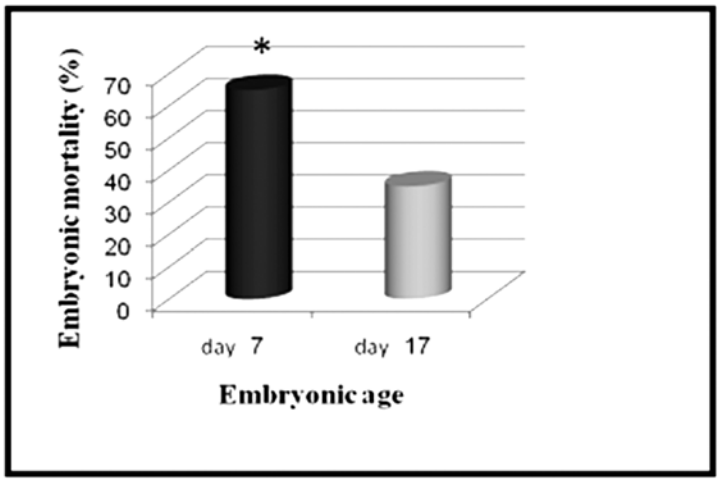


Figure- 2: Diagrams showing the effect of µm 0.02 (MTX) on the rate of embryonic mortality in the 7th and the 17th (C) days of gestational period.
(Chi2 value = 4.978), (P-value =0.026) column with star was significant different (p<0.05).
* Means within each columns is significantly different (P<0.05).

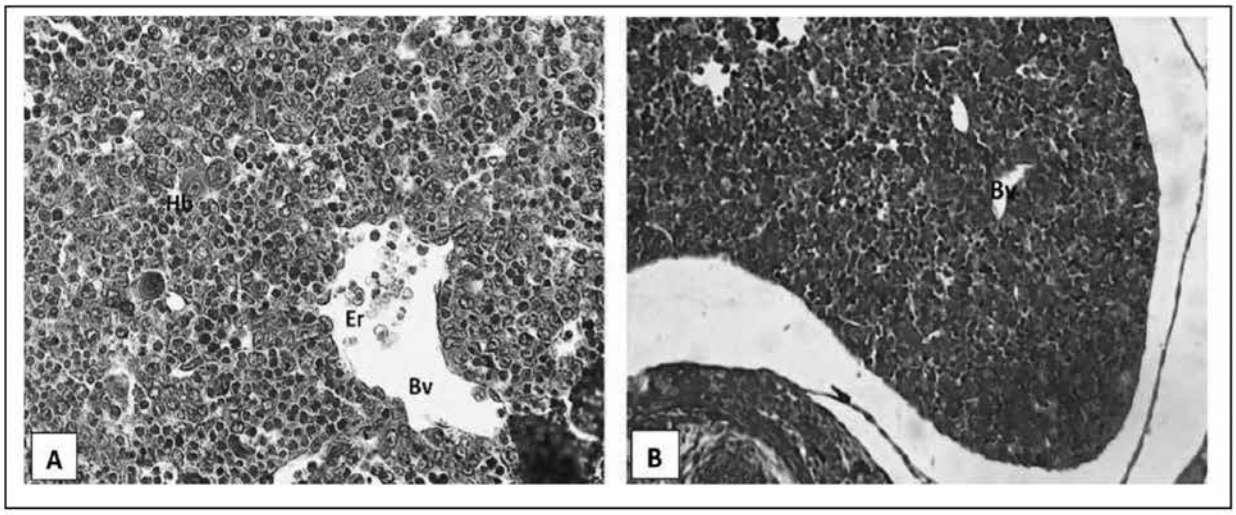


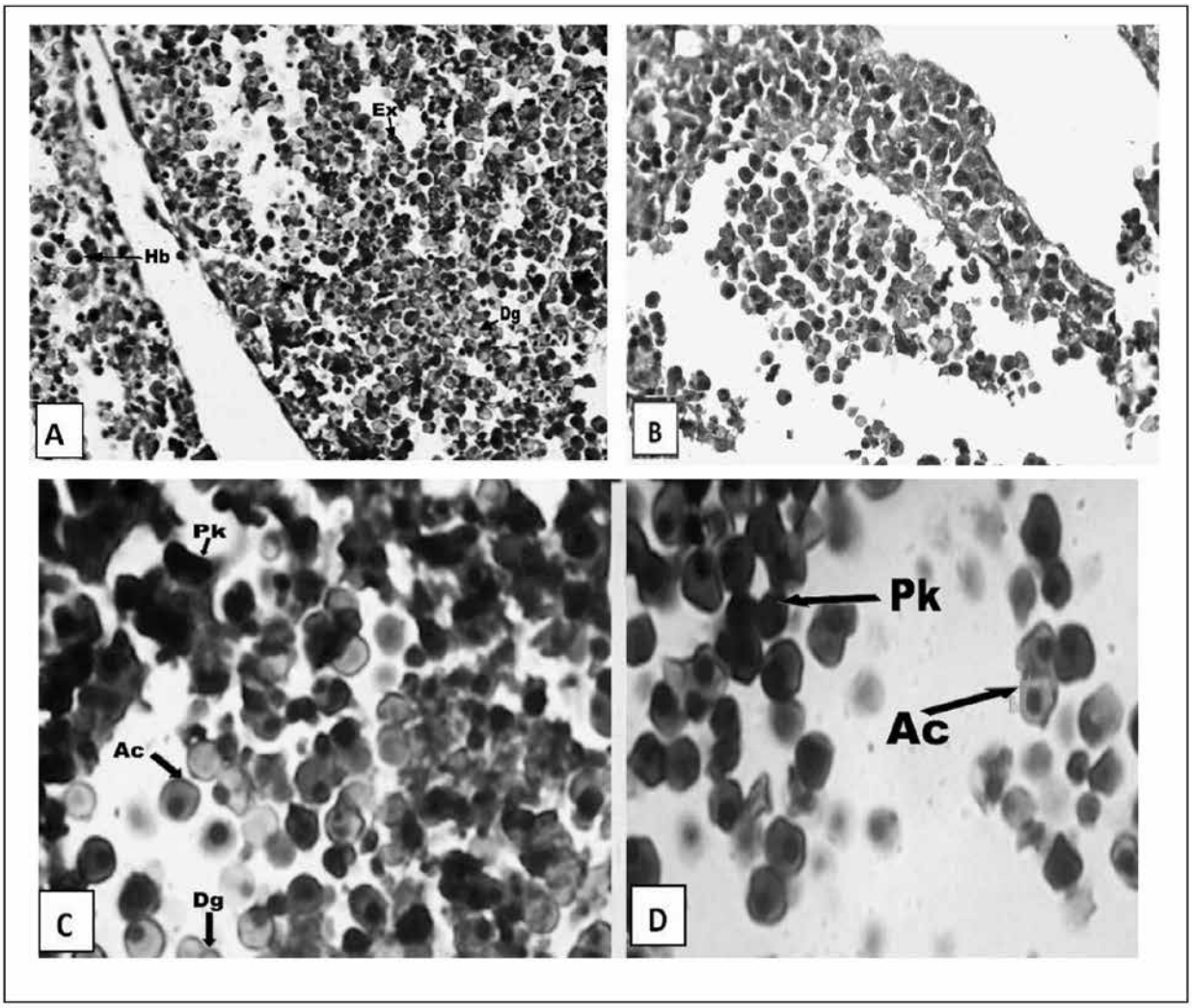
Figure- 3: Liver of control rat on the 7th (A) and the 17th (B) day's gestation showing normal architecture of hepatic tissue with developed hepatoblast "Hb". Er; Erythroblast, Bv; Blood vessel. H&E, X200.

DISCUSSION

Methotrexate is used as an antirheumatic agent and generally administered in low-dose to patients with rheumatoid arthritis and other rheumatic diseases; (Cronstein, 2005). It is also considered as successful drug in cancer chemotherapy and has been used for many years in treatment of various types of cancer and autoimmune disorders (Blinova *et al*., 2008).

Methotrexate been reported to cause fetal death or congenital defects when administered to a pregnant woman (Lloyd *et al*., 1999). In the present study, there is a highly significant increase (P< 0.01) in mortality in pregnant mice treated with MTX. Pervious study mentioned that the administration of MTX to normal animals might cause an increase in mortality rate if given for 9-day period. However, treatment with MTX for further time proved to be of high toxicity and all the mice died after 20 days on the drug regimen (Russell, 1972).

In the present study, the reduction in body weight was noticed in fetuses at days 7 and 17 of gestation. A highly significant increase (P< 0.01) in embryolethality was observed in mice treated with (0.02 mg/kg) of MTX as compared with control group. These results are in concordance with those Skalko and Gold (1972) who observed intrauterine death and malformations on the 17th day of gestation after a single intraperitoneal injection of 0.3-50 mg/kg MTX to ICR mice "Imprinting Control Region mice" and the intrauterine death rate increased on a dose of 10 mg/kg. Increased



nucleus (Ex) and others show degeneration (Dg), hepatic cells with apoptotic changes (Ac) and the hepatocyte loss its normal configuration with acidophilic cytoplasm appearance (Ac), hepatoblast "Hb". H&E, "A; X200, B; X400, C&D X1000.

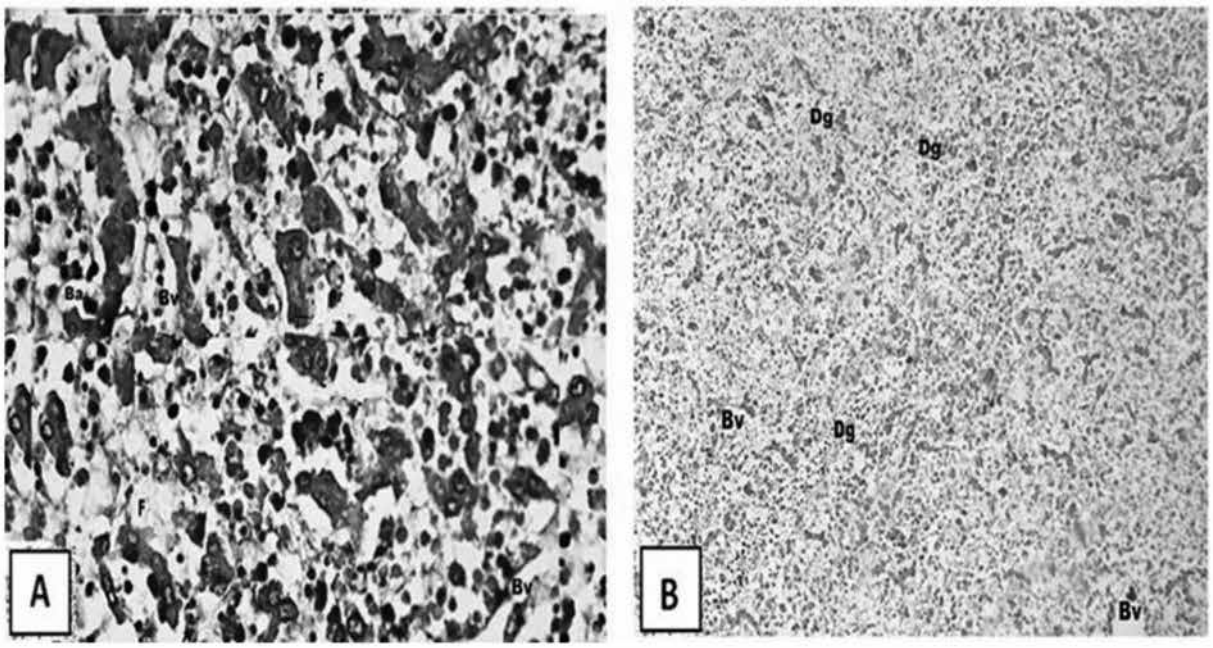


Figure-5: Liver of MTX treated rat on the 17th day' gestation showing the appearance of hepatic fatty change (F). Variability in nuclear size and staining, which ranged from minimal as pyknotic nuclei to marked pleomorphism and giant cells. Degenerated (Dg) and damaged sinusoid (Bv). H&E, A; X400, B X200.

embryolethality was also observed in mice treated intraperitoneally with 25 to 20 mg/kg MTX on the 8th day of gestation (Elmazar and Nau 1992).

The reduction of the mice embryos' weight in this study indicates that treatment with MTX during gestation lead to growth retardation. In the previous studies, the hepatotoxicity effects of the MTX has been demonstrated in biopsy specimens from cases treated with cumulative doses for long period(Curtis *et al*., 2010) and also in experimental animals such as pregnant rats(Jordan *et al*., 1977; Vardi *et al*., 2010), adult rabbits (Hadi *et al*.,2012), and mice (Baskerville and Cox, 1978).

In the present study, the histopathology of the embryonic livers showed varied changes from mild to severe fatty changes in all embryos treated with MTX. However, no cirrhosis was observed in the present study. A pervious study mentioned that there was very rare or absent cirrhosis in patients with definite or classic rheumatoid arthritis given methotrexate by the intramuscular route (Hoffmeister, 1983). The increased or cumulative dose of MTX reactions involves damage to hepatocytes throughout the hepatic lobule, with various degrees of necrosis, apoptosis and fibrogenesis (Lee, 2003).

Methotrexate undergoes hepatic and intracellular metabolism to polyglutamated conjugates, this process is reversible. Small amounts of the polyglutamates may be converted to 7-hydroxymethotrexate due to its low solubility, this hydroxyl metabolite may accumulate substantially following administration of high doses of MTX (Padmanabhan *et al*., 1986; Hadi *et al*., 2012).

Treatment with MTX in this study demonstrated liver fatty change. Long term therapy with methotrexate has been associated with mild fatty changes and periportal fibrosis of the liver (Hersh *et al*., 1966). Nonalcoholic fatty liver disease affects a large proportion of the world's population and may occur due to factors other than alcoholism, medication, obesity, malnutrition, starvation and rapid weight loss. Abnormal levels of lipids in the blood, high blood pressure or diabetes, insulin resistance, oxidative stress and genetic conditions might influence the fatty acid metabolism that have critical roles in the pathogenesis of nonalcoholic fatty liver disease(Chalasani *et al*., 2012).

The mechanism of MTX caused liver damage is unclear. Hepatic folate stores are depleted by MTX in the doses used in rheumatoid arthritis (RA), and these stores can be repleted by short-term administration of oral folinic acid (Rodenhuis *et al*., 1987). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are increased in patients with neoplastic diseases treated with MTX (Baskerville and Cox, 1978).

Immunohistochemical quantification of matrix proteins and collagens type III and IV are sensitive and dose responsive markers of MTX hepatotoxicity which progress with increasing cumulative doses of methotrexate (Jaskiewicz *et al*., 1996). *In vitro* and *in vivo* studies showed that MTX directly inhibit Methionine S-adenosyltransferase (MAT) mRNA expression and reduced MAT protein in the liver (Wang *et al*., 2012). MTX-induced reactive oxygen species (ROS) production and neutrophil infiltration and the DNA damage which resulted in small intestinal damage (Miyazono *et al*., 2004). MTX could affect the progression of inflammatory disease states by inhibiting monocyte interaction with the inflamed endothelium via the production of ROS (Phillips *et al*., 2003). The mortality, body weight loss and the liver histopathological alteration observed in this study clearly revealed the embryonic toxicity generated by the MTX during developmental changes and can affect internal organs causing damage during gestation period which lead to further birth defects in children born to women who have been treated with MTX. Further study of MTX on the kidney development is in progress.

CONCLUSION

The present study has demonstrated that administration of low dose MTX in early pregnancy has teratogenic effect on liver development.

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Effect of Cinnamon Extract on Blood Glucose Level and Pancreas Histopathology in Diabetic Rats

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ABSTRACT

The purpose of this research is to evaluate the effect of standardized cinnamon extract (CE) on fasting blood glucose (FBG) level and histopathological changes in islets of Langerhans in streptozotocin-induced diabetic rats (STZ). Twenty five rats were divided into five groups (n=6); normal control, normal treated with "CE" control, diabetic control (non-treated with "CE"), diabetic treated with 200mg/kg CE aqueous extract and diabetic treated with 0.5mg/kg glibenclamide (GLB). All the drugs were given orally as a single daily dose for 30 days. The FBG was determined once per week using Glucometer from rat tail vein.
On the first and second week, the 2 groups of diabetic rats treated with CE and GLB showed no significant differences of FBG as compared to control group. On the subsequent weeks only GLB-treated group showed no significant differences in FBG compared to the normal control. Histology observation revealed that both CE and GLB treated group showing restoration of the normal architecture of the islet structure compared with STZ untreated group which appeared as shrunken islet with degranulated β-cells, and pyknotic nuclei. It is concluded that both CE and GLB treated groups demonstrated significantly lower FBG than that of STZ group. CE proved to be effective in reducing FBG in diabetic rats, but it was less potent than GLB.

Keyword: Diabetes Mellitus, Streptozotocin, cinnamon, Glibenclamide, Islets of Langerhans

INTRODUCTION

Diabetes mellitus DM is metabolic disorder characterized by abnormally elevated blood glucose (Albajali al., 2011). Lack of insulin secretion or combination of insulin resistance and insufficient insulin secretion to utilize the glucose in the blood are the factor that contribute to T2DM (Kamble and Rambhimaiah, 2013). Streptozotocin (STZ) is a naturally occurring chemical produced by Streptomyces acromogenes, which is particularly has a high specific cytotoxic action on the β cells of the pancreatic islets (Al-Badri *et al*., 2011; Brunton and Chabner, 2011). It has been shown in animal's model to induce a chronic diabetic state resembling human hyperglycemic DM as it develops many features seen in human patients (Yuan-yuan and Shan-dong, 2013).

Nowadays, common synthetic drugs from sulfonylurea and biguanide groups are available in the market for T2DM treatment, they are mainly used to control normal blood glucose in order to maintain the glucose level in the normal range and to prevent or delay serious diabetic complications (Sailesh and Padmanabha, 2014). Unfortunately, up until now, none of them are clinically proven to completely cure diabetes mellitus (Alese *et al*., 2014). They are only capable of reducing elevated blood glucose and protecting the patients from the risk of macrovascular and microvascular complications (Kumar *et al*., 2013). However, these drugs may cause many side effects, the patients may develop resistance, and they do not adequately inhibit the development of the associated complications like causing hypoglycemia at higher doses, liver problems, lactic acidosis and diarrhea (Zia *et al*., 2001; Halim, 2003; Al-Ani *et al*., 2009).

Currently, many herbal medicines therapeutic have been recommended for the treatment of diabetes and its associated complications; they are recommended widely because of their helpfulness, less side effects and relatively low cost (Venkatesh *et al*., 2003; Al-Badri *et al*., 2011). Cinnamon is one of the most widely used flavouring agents used in the food and beverage industry

worldwide and well recognized for its medicinal properties since antiquity (Medagama and Bandara, 2014). Cinnamon has biologically active ingredients with insulin mimicking properties (Qin *et al*., 2003). It has been postulated that cinnamon leaves may play a role in the prevention and treatment of type 2 diabetes (Khan *et al*., 2003). The main objective of this study is to evaluate the antidiabetic effect of cinnamon extract (CE).

MATERIALS AND METHODS

Cinnamon

Standardized CE was obtained from Cinnaba® product which already prepared in capsule form. The extract is standardized to contain 0.95% of trimeric and tetrameric polymers and 1% corosolic acid which aids in absorption. The extract granule then was added with distilled water and mixed together using hot plate stirrer. After 10 minutes of mixing, the solution was filtered to obtain the cinnamon extract.

Animals and experimental design

25 Sprague Dawley rats ranging in weight from 150 - 200g were used. After 1 week of acclimation, under the standard laboratory conditions (adequate cross ventilation; temperature: 24 ± 2°C; 12:12 hrs light: dark cycle; relative humidity: (46-79%), the rats were maintained on standard commercial dry pellet diet and water ad libitum. and were randomly divided into five groups of 5 animals in each group as described in Table 1.

Table 1. Distribution of rats into groups according to the treatment.

Group	Definition	Treatment
Group 1	Normal control rats	Rat pellets and water only
Group 2	Normal CE treated control rats	Rat pellets, water & fed with CE-200mg/kg
Group 3	Diabetic control rats	STZ 50mg/kg, + pellets and water
Group 4	Diabetic rats treated with CE	STZ 50mg/kg, CE-200mg/kg + pellets and water
Group 5	Diabetic rats treated with Glibenclamide	STZ 50mg/kg, Glibenclamide 0.5 mg/kg + pellets and water

The rats were fasted overnight (12-14 hours). The animals were given a single intraperitoneal STZ injection of 50 mg/kg of body weight dissolved in citrate buffer PH 5.4 In order to prevent hypoglycemia; normal control rats received an equivalent volume of distilled water. Three days post induction, Accu-Chek Performa glucometer was used to measure the level of fasting blood glucose. Only rats with high level of fasting blood glucose (200mg/dL) were considered as diabetic rats. Syringe and special designed metal ball-ended needle were used to feed the rats daily by gavage. Groups (2 and 4) received 200mg/kg of standardized CE orally. Groups 5 rats were fed with glibenclamide 0.6 mg/kg.

Histology Examination

All rats were sacrificed at the 30 days of experiment; the animals were sacrificed using Ketamine as an aesthetic. Pancreatic specimens were rapidly removed, fixed in 10% formal saline for 72 hours, dehydrated through graded alcohols and cleared using two changes of xylene and embedded in paraffin wax. Sections of 4-5 micron thickness were prepared using the microtome, stained Haematoxylin and eosin (H & E),

Statistical Analysis

Mean ± SEM was used for statistical analysis by means of SPSS 21.0 software. Repeated Measure ANOVA Test was used to evaluate the reduction of blood glucose as compared to the control group. P value of less than ($P<0.05$) was considered statistically significant and $P>0.05$ not significant.

RESULTS

Antihyperglycemic effect of CE aqueous extract

The basal mean FBG levels for all groups of rats were not statistically different from each other; treatment of the rats with CE didn't show differences from the normal control rats throughout the period of the experiment, the normal control groups and those treated with CE showed persistent normoglycaemic values throughout the course of the study. However, administration of STZ elevated the FBG values three to five folds higher ($p < 0.000$) when compared to normal controls. Treatment of STZ diabetic rats with standardized CE exhibited statistically significant decrease in FBG level ($P < 0.15$). On the other hand, STZ diabetic rats treated with GLB showed significant reduction ($P < 0.000$) in the basal mean FBG levels (Figure 1).

Histological observation

The islets of Langerhans of both control CE and treated groups have normal in histological architecture; they were unevenly distributed in the exocrine pancreatic tissue and they were of varying sizes in the same pancreatic lobule (Figures 2 and 3). The STZ diabetic rats showed shrunken islets with degranulated β-cells, and pyknotic nuclei, the endocrine cells were separated by empty spaces and congested blood capillaries (Figure 4). Treatment with CE extract and with Glibenclamide to STZ diabetic rats stimulated significant improvement in degenerative changes induced by STZ injection in endocrine pancreas, the islets showed normal looking appearance (Figures 5 and 6).

DISCUSSION

The objective of the present study is to investigate the hypoglycemic effect of Cinnamon extract in STZ diabetic male rats. The present study has demonstrated hyperglycemia three days after STZ administration and directly treated daily with graded dose (200 mg/kg) of CE aqueous extract for 30 days, the result showed that the CE produced a significant decrease in fasting blood glucose (FBG) level compared to diabetes untreated group. These findings are in agreement with Kamble and Rambhimaiah (2013) who concluded that after 15 days of treatment with aqueous extract of cinnamon extract (60mg/kg), showed a significant in FBG level on 10^{th} and 15^{th} day of study. Oral administration of cinnamon for 21 days decreases blood sugar level in diabetic rats induced by alloxan (Kumar and Mukkadan,2013; Sailesh and Padmanabha, 2014). Cinnamon extract administration has been found to induce beneficial effects in STZ diabetic rats and affect the genes related to carbohydrate and lipid metabolism suggesting insulin like effects (Soliman *et al*., 2011).

Histopathological examination of pancreas sections from STZ control rats of this study is agreed with Al-Badri *et al*. (2011) and Al-Ani *et al*. (2016) who showed that there was extensive damage of the Islets of Langerhans. It is well known that the β cells of the pancreatic islets are responsible for insulin production, exhaustion and reduction of β cells will therefore result in insulin deficiency which will lead to a disorder in carbohydrate, protein and fat metabolism with a resultant hyperglycaemia. In the present study, treatment of rats with 50mg/kg STZ was adequately enough to induce severe damage on pancreas, resulting in reduction of pancreatic islet area and increase in atrophied area compared to control ones., resulting in reducing the function of Langerhans islet beta cell to produce insulin. The mechanism of action of streptozotocin (STZ) is via the destruction and degeneration of β bells of the pancreatic islets which results glucose oxidation

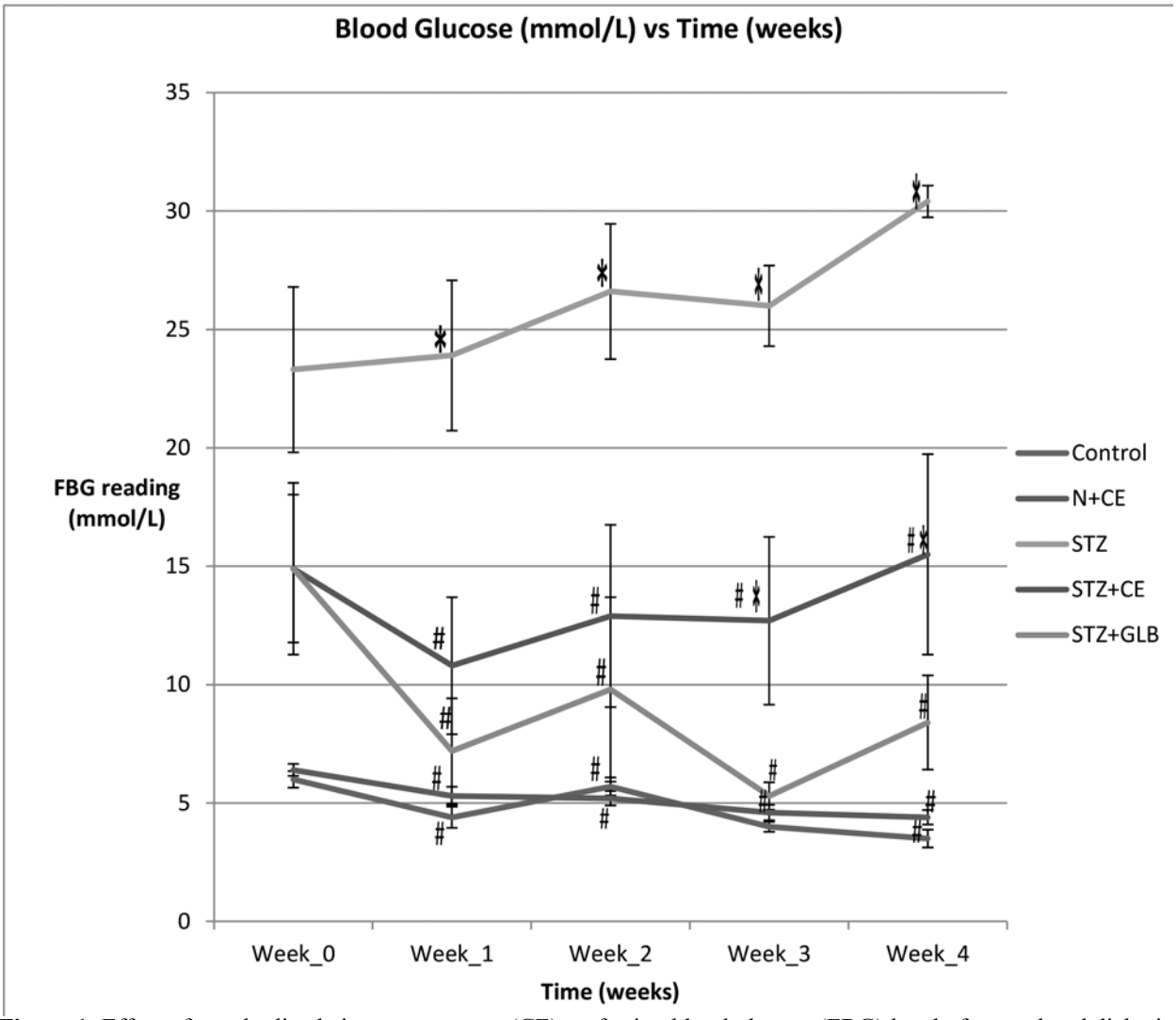

Figure 1: Effect of standardized cinnamon extract (CE) on fasting blood glucose (FBG) level of normal and diabetic rats induced by streptozotocin (STZ). (* = $P < 0.05$ vs control group). (# = $P < 0.05$ vs STZ group).

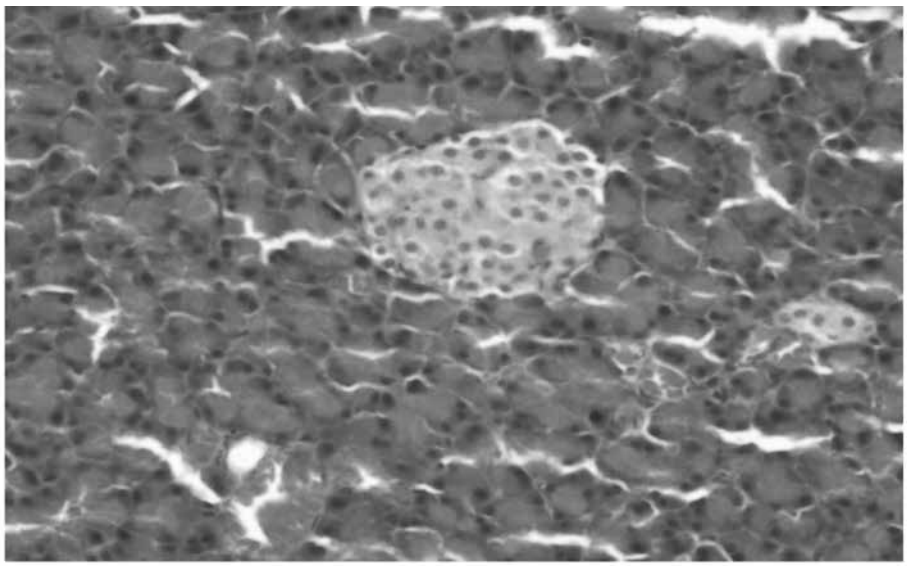

Figure 2: Islet of Langerhans of normal non-diabetic rat showing islet cells arranged as anastomosing cords profusely by fenestrating capillaries (H & E ×400).

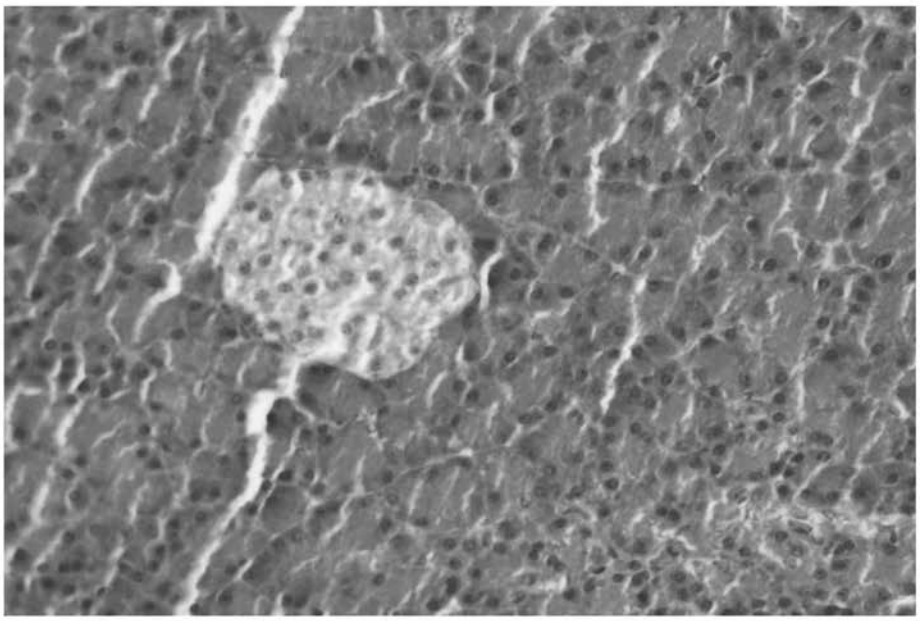

Figure 3: Islet of Langerhans of Cinnamon treated rats showing normal islet cells arranged as anastomosing cords perfused by fenestrating capillaries (H& E ×400).

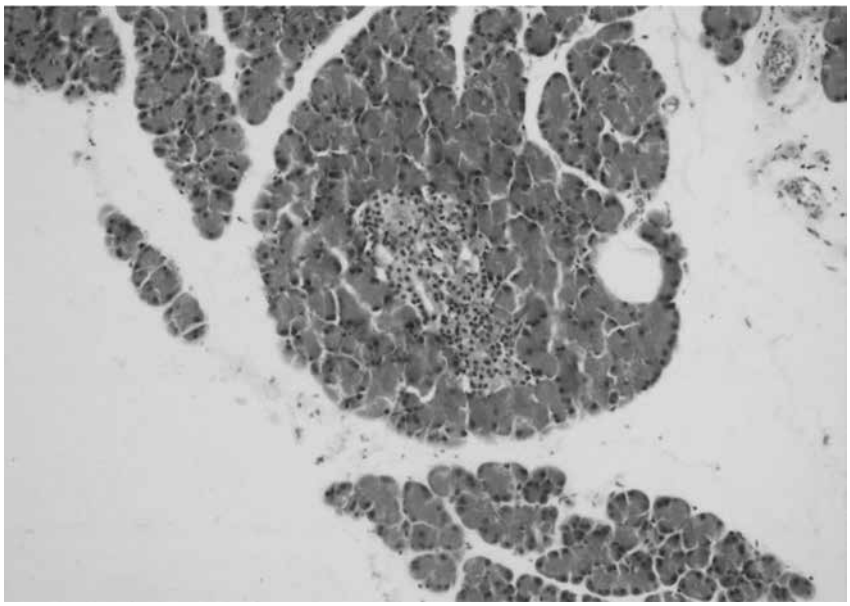

Figure 4: STZ diabetic rat showing shrunken islet with degranulated β-cells, and pyknotic nuclei (H& E ×400).

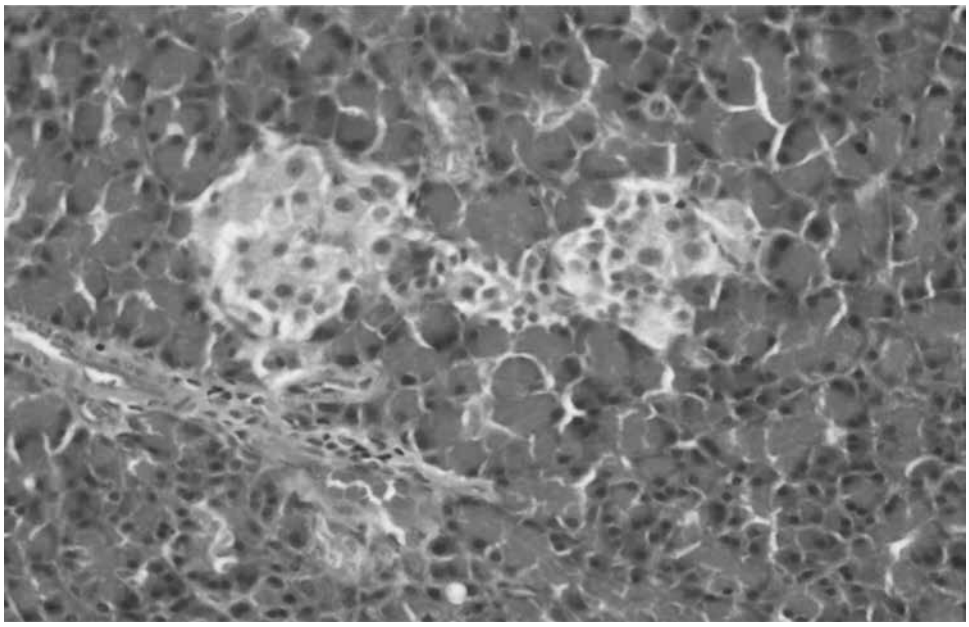

Figure 5: Pancreas of STZ diabetic rat treated with Cinnamon Extract showing normal looking appearance of the islet structure. (H& E ×400).

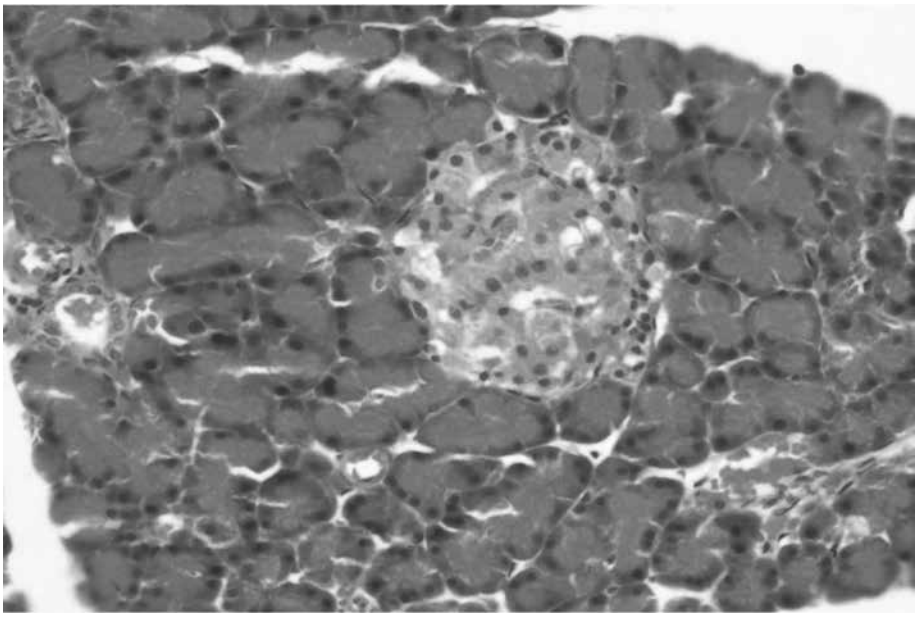

Figure 6: Pancreas of STZ diabetic rat treated with Glibenclamide showing restoration of the normal architecture of the islet structure (H& E ×400).

impairment and low serum insulin level (Akbarzadeh *et al*., 2007; Al-Badri *et al*., 2011). In the present study, treatment of the diabetic islets with either dose (200 mg/kg) of CE aqueous extract and (0.5mg/kg) glibenclamide for 30 days showed improved islet architecture; this was associated with the improved glucose levels. However glibenclamide was more potent hypoglycaemic agent than cinnamon.

The precise mechanisms of the ameliorative effect of cinnamon extract on the histopathological changes and blood glucose level in STZ-induced diabetic rats are inconclusive and inadequate data are available to make any generalized conclusion. However, there are four active ingredients that contribute to a decrease in blood glucose level when tested on rat which are methylhydroxy chalcone polymer with insulin mimetic properties (Khan *et al*.,2003; Kim *et al*., 2006). CE also contains procyanidin-B2, cinnamaldehyde and polyphenol compound of cinnamon (Shihabudeen *et al*.,2011) and demonstrated hypoglycemic effect in rats (Qin *et al*.,2004). According to Kamble and Rambhimaiah (2013), the most important component in cinnamon is methyhydroxy chalcone polymer (MHCP) which has been proved to exert insulin action in isolated adipocytes based on numerous in-vitro studies. This component is present in the bark and has the ability to increase glucose metabolism and stimulating cell sensitivity to insulin (Shihabudeen *et al*.,2011).

As stated by Khan *et al*. (2003), one of the major role of MHCP is by amplifying the expression of peroxisome proliferator-activated receptors y and a (PPARy/a)7,8, improves glucose metabolism and glucose uptake by water soluble polyphenol polymers, resulting in the increase of pancreatic secretion of insulin from beta cells. According to Mahmood *et al*., (2011), 200mg/kg of cinnamon showed significant glucose lowering effect from the period of 0-6 weeks. The findings also indicated that FBG level was significantly decreased ($P < 0.05$) with the 100mg/kg dose of CE group ($P < 0.01$) compared to diabetic control group.

GLB is one of the oral hypoglycaemic agents from sulfonylurea group that have been used nowadays to treat diabetes mellitus. The mechanism of action of the glibenclamide lead to inhibition of the ATP-sensitive potassium channels, which results in depolarization of the cells and insulin secretion (Ashcroft, 20). GLB works mainly by stimulating the cells in the pancreas that produce insulin which are called beta cells. It causes the beta cells to produce more insulin, thus helps to decrease the amount of glucose in the blood. Further electron microscopic investigations on the pancreatic islets of STZ diabetic rats treated with cinnamon are in progress.

CONCLUSION

It is concluded from the present study that daily treatment with cinnamon extract shows hypoglycaemic activity and protective effect in STZ diabetic rats indicating that cinnamon extract represents a candidate alternative treatment to control diabetes mellitus and its related pathological changes.

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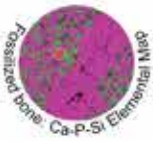
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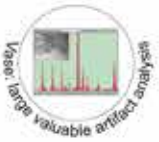
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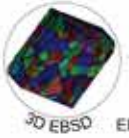
3D EBSD


EDS, WDS, EBSD


EPMA


In-situ dating of monazite


WDS Large Area Boron Map


WDS Spectrum of Monazite


UHR imaging (catalyst)


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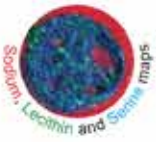
3D Al2+GaAl Distribution


Sodium, Lecithin and Sterile maps


Surface Spectroscopy of Polystyrene

BF-STEM of catalyst Cell

Gate-oxide on Si substrate

C_s corrected STEM BF, GMR head

C_s corrected STEM

LEXES

B Dose Map

Dose Uniformity

Quantitative Dose Measurement

Carbon Isotope in Diamond

Geochronology - U/Pb Dating

Phosphorous Depth Profile

Sector SIMS

FIB

High quality TEM lamellas

High speed FIB milling

Micro-machining

3D Atomic structure of SiO_2

Atomic Precipitation in Super-alloy

Tunnel Barriers Fe-MgO-Fe

TAP

LEIS

ALD Cycles of WNxCy

Mo-V-Te-O catalyst

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