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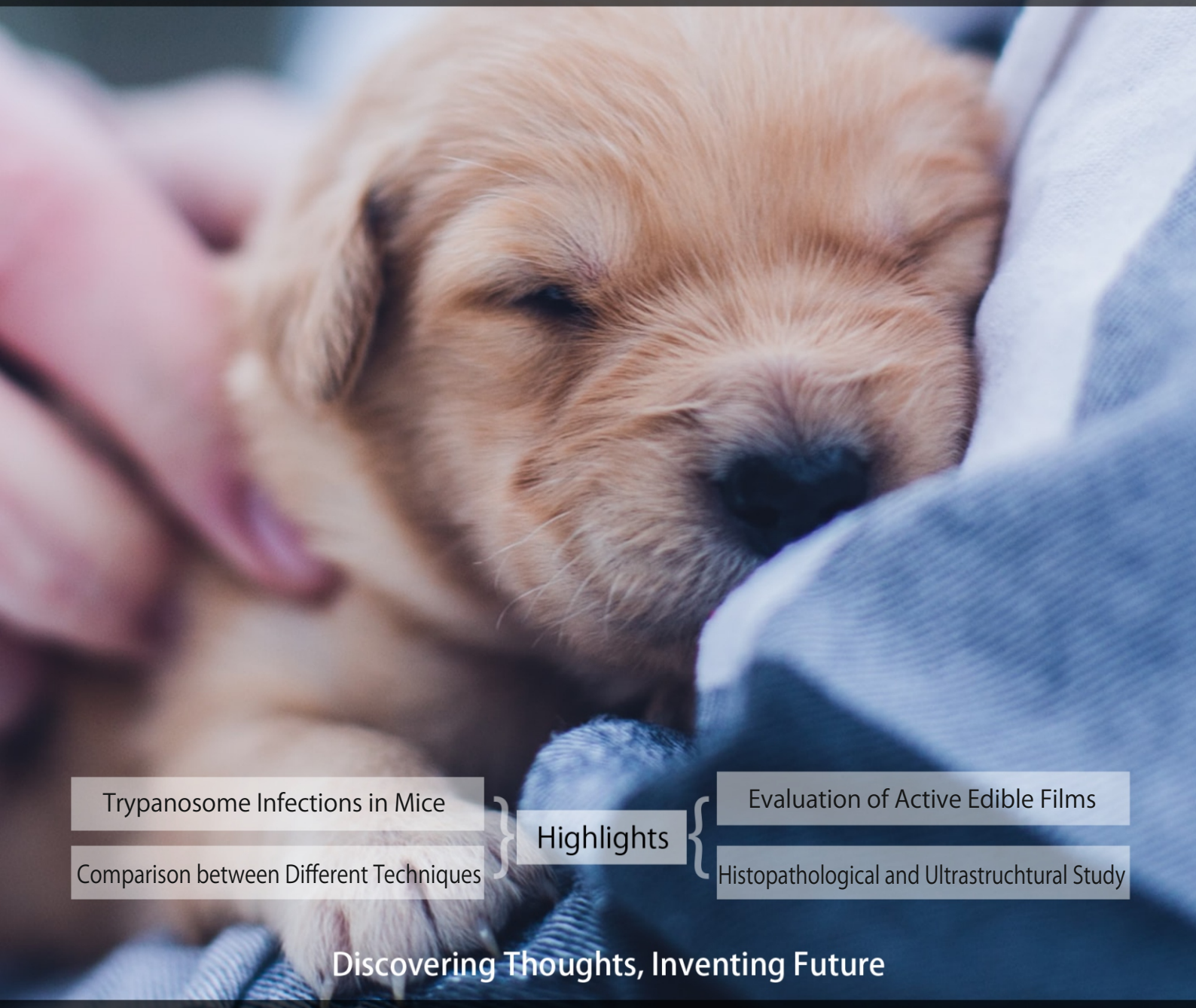

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Highlights

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Discovering Thoughts, Inventing Future

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Protective Effect of Propolis against Aluminium Chloride Reproductive Toxicity Histopathological and Ultrastruchtural Study

By Essam Eldin Abdelhady Salama
King Saud University

Abstract- Human impact on the environment was steadily increasing the amounts of aluminum in the ecosystems. This element accumulated in plants and water. Potentially increased its harmful effect; particularly, it induced free radical-mediated cytotoxicity and reproductive toxicity. Propolis was a resinous material collected by bees from bud and exudates of the plants, mixed with bee enzymes, pollen, and wax. Due to its waxy nature and mechanical properties, bees used propolis in the construction and repair of their hives, and as a protective barrier against external invaders, or weathering threats, Current antimicrobial properties of propolis, was helping for wound healing, treatment of burns, herpes simplex and genital herpes. The present work studied the protective effect of propolis against the reproductive toxicity of aluminum chloride in male rats.

Keywords: *male albino rat. Aluminum chloride toxicity. Propolis.*

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Strictly as per the compliance and regulations of:

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Abstract- Human impact on the environment was steadily increasing the amounts of aluminum in the ecosystems. This element accumulated in plants and water. Potentially increased its harmful effect; particularly, it induced free radical-mediated cytotoxicity and reproductive toxicity. Propolis was a resinous material collected by bees from bud and exudates of the plants, mixed with bee enzymes, pollen, and wax. Due to its waxy nature and mechanical properties, bees used propolis in the construction and repair of their hives, and as a protective barrier against external invaders, or weathering threats, Current antimicrobial properties of propolis, was helping for wound healing, treatment of burns, herpes simplex and genital herpes. The present work studied the protective effect of propolis against the reproductive toxicity of aluminum chloride in male rats.

Material and Methods: Sixty male Wistar Albino rats (average weight 200 g) were used and divided into three groups; each contained twenty rats. Groupe I, was a control group, group II, was treated with aluminum chloride (80 mg / Kg. body weight) through an orogastric tube, daily for six weeks. Group III, was given aluminum chloride (80 mg / Kg. body weight), concomitant with (200 mg / Kg. body weight) ethanol extract of propolis, for the same root and period.

Results: Study of the second group revealed degeneration and necrosis of the seminiferous tubules, as demonstrated with the light microscope. The ultrastructure study revealed dilated irregular tubular membrane, Sertoli cell, spermatogonia, and early spermatid with vacuolated cytoplasm and irregular nuclei, swollen mitochondria, dilated irregular tubular membrane. Apparent improve in the light and ultrastructure study of the third group. The results concluded the administration of aluminum chloride to adult male rats would cause adverse effects on reproductive efficiency that could be overcome by the protective effect of propolis.

Objective: Of our results would suggest that propolis would provide a therapeutic value against aluminum-induced male reproductive toxicity.

Keywords: male albino rat. Aluminum chloride toxicity. Propolis.

I. Introduction

Aluminum was widely spread in our environment. It was the third most abundant element on the earth's crust [1]. It has no known biological role in living organisms and may be classified as toxic metal [2]. It was widely used for the manufacturing of many products [1]. And as a result, food consumption was the major source for aluminum intake under physiological conditions [3]. The widespread presence of aluminum, both in the environment and in food, made it almost impossible to avoid exposure to this metal ion [4]. Aluminum -containing diet was mainly corn, yellow cheese, salts, herbs, spices, and tea, [5]. It entered in production of cosmetics such as antiperspirant and deodorant preparation [6]. Moreover, it incorporated in some medications such as antacids, buffered aspirin, antidiarrheal products, vaccine, and allergen injection [7], [8], and [9]. Also, it used as a component of veterinary medicine, glues, and disinfectants [10]. Aluminum sulfate added as a coagulant agent during the purification process of drinking water [11]. Recently, Aluminum in drinking water led to the development of many diseases and health problems [1]. Aluminum chloride was suggested to induced reproductive toxicity and exerted a significant adverse effect on steroidogenesis [12]. Aluminum compound had serious effects on energy metabolism and hematology [13], [14]. It enhanced free radicals, and changed the enzymatic activities [15], with emphasis on the alterations in the metabolism of testis, epididymis and vas deferens that led to poor sperm motility and reduction in fertility rate in mice [16],[17]. It reduced fertility and reproductive activity [18],[19], and also affected the process of development of embryos by its embryotoxicity and teratogenicity [20]. Liobet et al. [21], Khattab [22], Guo et al. [23], and Fairoze [24]; observed histological changes including necrosis of spermatocytes and spermatids in the testis of male mice treated with aluminum nitrate, and degeneration in the structure of spermatogenesis and formation of giant cells in mice testis treated with aluminum chloride, severe damage within seminiferous tubules and vascular degeneration on spermatogenic and Sertoli cells cytoplasm.

Propolis was a natural resinous mixture produced by honeybees from substances collected from parts of plants, buds, and exudates. Due to its waxy nature and mechanical properties, bees used propolis in the construction and repair of their hives, sealing openings, and cracks, smoothing out the internal walls, and as a protective barrier against external invaders like snakes, lizards, winds and rains

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[25], [26]. It possessed a characteristic and pleasant aromatic smell and various colors from transparent propolis, yellow, green, red, or dark brown, depending on the site of origin, source, and age of the resins, it was hard and brittle, and became soft, pliable, gummy, and very sticky when heated [27]. Raw propolis was composed of around 50% resins, 30% waxes,10% essential oils, 5% pollen, and 5% of various organic compounds [28], [29], and [30]. Propolis was soft, pliable, and sticky substance at 25∘C to 45∘C, above 45∘C; it became gummy, and liquid at 60∘C to 70∘C, but for some samples the melting point might be as high as100∘C [27]. Propolis was extracted with a suitable solvent, the most common solvents used for extraction were water, ethanol, methanol, chloroform, ether, and acetone [31]. According to Ugur and Arslan [32], the use of different solvents changed the biological activity of the main active constituent in propolis. The aqueous extract of propolis showed higher antioxidant activity compared to the ethanol extract of propolis; this was due to its higher polyphenols content. So, it used in prevention of various free radical related diseases. Ethanolic extracts of propolis showed high antibacterial activity against Gram-positive, but least activity against Gram-negative bacteria [33], [34]. Selvan et al. [35] reported that bee propolis, in combination with chlorhexidine, possessed high antimicrobial activity against streptococcus mutants. Propolis was the bee product with the highest antifungal activity as tested with 40 yeast strains of C. Albicans [36],[37]. Propolis showed fungicide effects that prevented juice spoilage [38]. According to Dota et al. [39]. The propolis extract had fungicidal effect against clinical yeas, and also for of the vulvovaginal candidiasis. The European propolis cured some human diseases caused by protozoa [40],[41] and [42]. Propolis was known for its antioxidant properties [43], [44]. The antioxidants present in propolis played a great role in its immunomodulatory properties [45]. All propolis had strong antioxidant activity due to their contents of phenols and flavonoids which increased the cellular immune response [46]. Active flavonoids and cinnamic acid derivatives were responsible for the anti-inflammatory activity of propolis [47], [48], [49], and [50]. Propolis provided beneficial effect on human health, due to its antimicrobial, antiviral, and antioxidant properties, it was widely used in human and veterinary medicine. It entered in manufacturing of cosmetics, medication for cold syndrome, and flu-like infections, treatment of wounds, burns, and acne. It was commercially available in the form of capsules, creams, mouthwash solutions, toothpaste, and, throat lozenges [27].

II. Material and Method

Chemicals: Aluminum chloride (AlCl3) came from Guangdong Guanghua Sci-Tech Co. Ltd., Shantou, Guangdong, China, and manufactured by Yueyang Jiazhiyuan Biological Co. Ltd., China.

The dose of aluminum chloride was 80 mg/kg. orally, daily, for six weeks [51].

An ethanol extract of propolis came from Boiron et Cie, Lyon, France.

The dose of the ethanol extract of propolis was 200 mg/kg. orally, daily, for six weeks [52].

Experimental design: Sixty male rats of proven fertility divided into three groups.

Group I: Control group, rats received 2.0 ml. distilled water orally, daily, for six weeks.

Group II: Treated group, rats received aluminum chloride 80 mg / Kg. Orally, daily, for six weeks, it was 6 mg. Dissolved in 1.0 ml. Distilled water.

Group III: Treated group, rats received aluminum chloride as the same dose, root and period for group II, and 200 mg/kg. Ethanol extract of propolis, orally, daily, for six weeks, it was about 40 mg. Dissolved in 1.0 ml. Distilled water. After the designed period, the testes were dissected out, trimmed off the attached tissues. Specimens of the testes fixed immediately in 10% buffered formalin for histological study.

For Histopathological and Ultrastructural Examination: According to Bancroft and Gamble [53], tissues were taken from testes of rats were cut and immediately fixed into 10% formaldehyde saline. Tissues were processed for the preparation of paraffin blocks (paraffin method). Sections were cut by rotatory microtome and mounted on glass slides. The sections stained by conventional Hematoxylin and Eosin (H&E), PAS, and Mallory stain, and examined by light microscope.

For the ultrastructural examination: An adjacent section of testis was removed and placed in 0.1M cacodylate buffer containing 3% glutaraldehyde for electron microscopy. After fixation, sections were dehydrated in a series of ethanol rinses, cleared with propylene oxide, and embedded in Epon. (*Epon or Epon-Araldite* mixtures are the most widely used resins for electron microscopy.) The sections were evaluated using a transmission electron microscope (Joel 1200 Ex-II). [53].

III. Results

I Histological study

Group I: Control group.

The seminiferous tubules were well circumscribed with a clear basement membrane. The germ cells arranged in multiple layers with the sperms occupied the lumen. The interstitial tissue filled the spaces between the seminiferous tubules, which contained blood vessels, and the interstitial cells of Leydig, which appeared in groups of polyhedral cells (Fig.1-A). The Mallory stain showed the red interstitial

tissue and the normal thickness of the tunica albuginea (Fig.2-A). The PAS stain showed normal thickness and regular pattern of the basement membrane (Fig.3-A).

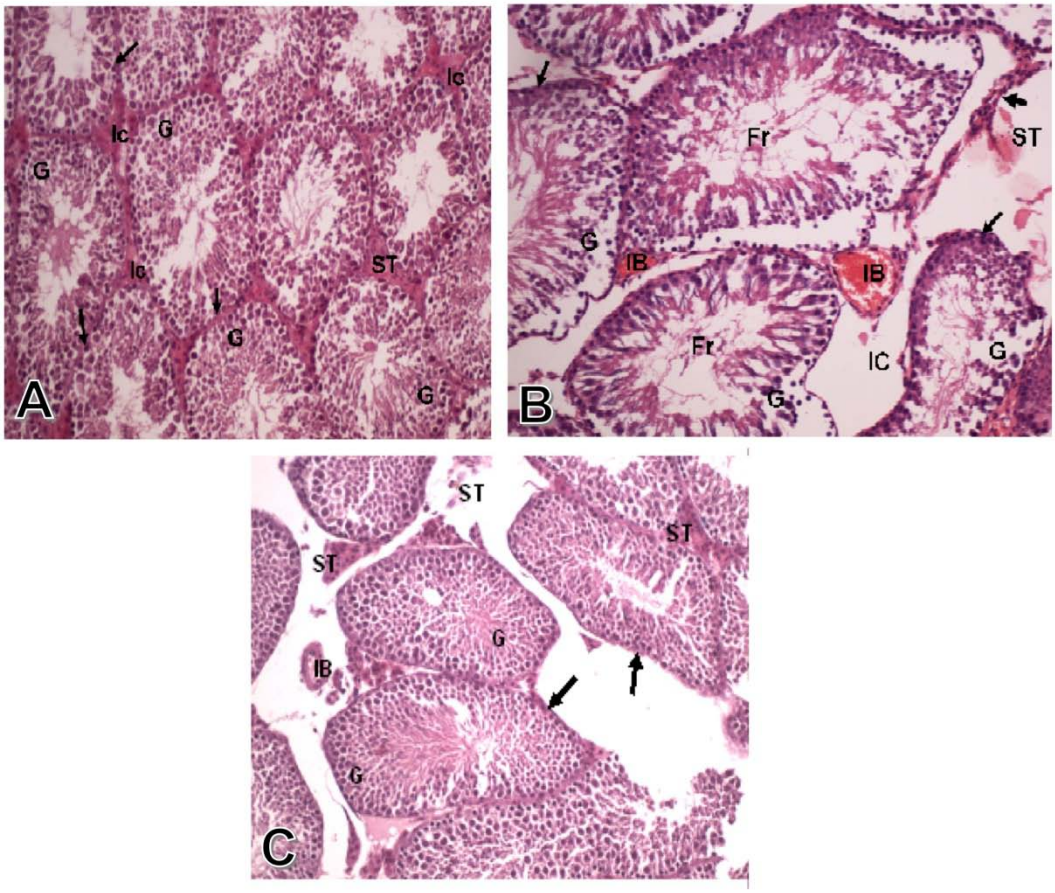

FIG. 1: **(A)** Photomicrograph of transverse section of testis of a control rat, showing the different stages of spermatogenic cells (G) the seminiferous tubules with thin normal basement membrane (arrow), and the interstitial cells (ST). (H&E X 250). **(B)** Photomicrograph of transverse section of testis of the treated group with alumimium chloride, showing marked degeneration and necrosis of germ cells (G) lining of the seminiferous tubules with thick basement membrane (arrows), and fragments of germ cells (Fr) within the lumen of seminiferous tubules. There are also edema of interstitial cells (IC), and congestion of interstitial blood vessel (IB). (H&E X 250). **(C)** Photomicrograph of transverse section of testis of the treated group with alumimium chloride, and propolis, showing nearly normal histological pattern in the germ cells (G) lining the seminiferous tubules with normal basement membrane of that tubules (arrows), as well as normal interstitial cells of Leydig (ST) and no congestion of interstitial blood vessels (IB). (H&E X250)

Group II: Treated with aluminum chloride.

Some of the seminiferous tubules showed marked degeneration and necrosis of the germ cells lining of the tubules. The degenerated tubules showed thick basement membrane and fragments of germ cells in the center of the tubules. There was also edema between the interstitial cells and congestion of interstitial blood vessel (Fig.1-B). There was some fibrosis of the interstitial tissue which appeared blue with Mallory stain (Fig.2-B). There was a decreased PAS reaction in the markedly degenerated germ cells lining the seminiferous tubules, and the interstitial cells (Fig 3-B)

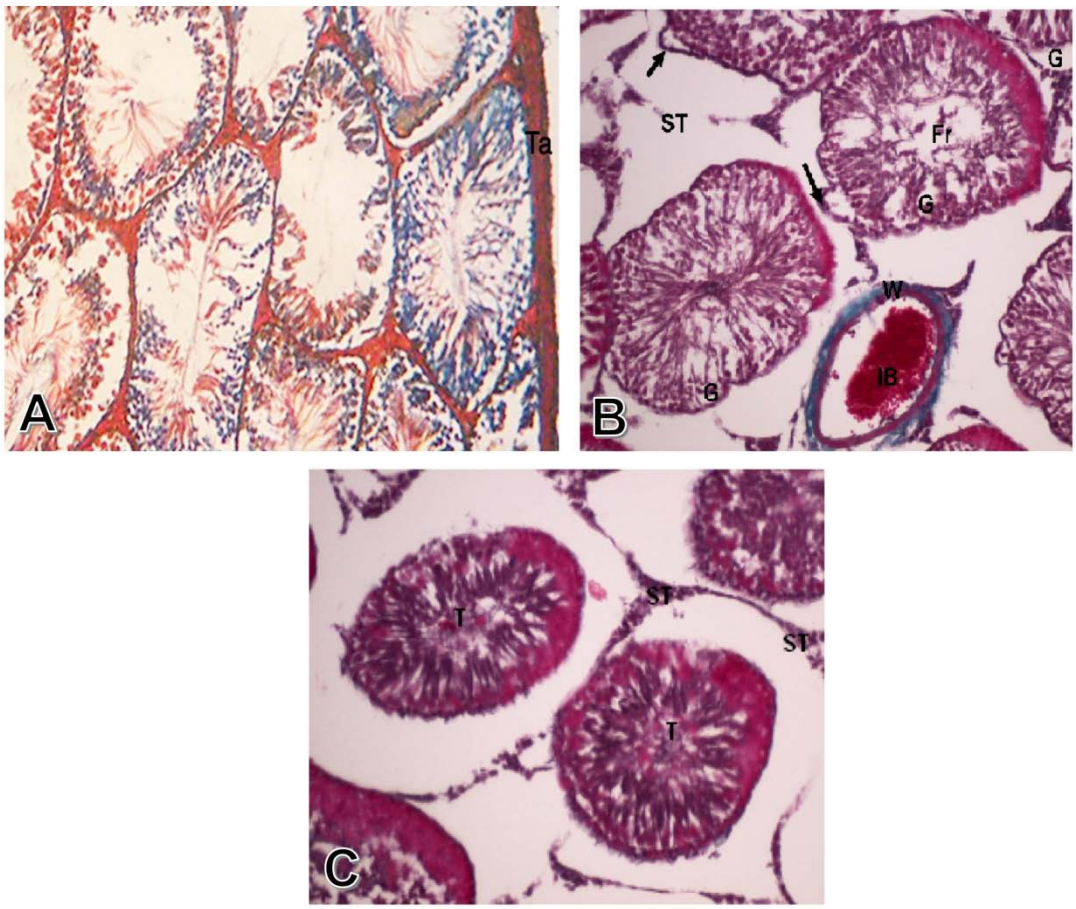

FIG. 2: (A) A photomicrograph of a transverse section of a control rat testis showing normal thickness of tunica albuginea (Ta).(Mallory X250) (B) Photomicrograph of transverse section of testis of the treated group with alumimium chloride, showing congestion of interstitial blood vessel (IB) with necrotic wall (W), as well as degeneration and necrosis of germ cells (G) lining the seminiferous tubules with fragments of germ cells(Fr) and thickening of tubules basement membranes (arrows).(Mallory X250) (C) Photomicrograph of transverse section of testis of The group treated with alumimium chloride, and propolis, showing normal histological pattern of seminiferous tubules (T) and normal interstitial cells (ST). (Mallory X250).

Group III: Treated with propolis with concomitant administration of aluminum chloride.

The testis of rats treated with aluminum chloride and propolis extract, showed normal histological pattern of the germ cells, basement membrane, and interstitial cells of Leydig, with lack of congestion of interstitial blood vessels (Fig.1-C).The Mallory stain showed normal histological pattern of seminiferous tubules and the interstitial cells (Fig. 2-C). There were marked increase in PAS reaction of the seminiferous tubules components, and the interstitial cells of Leydig (Fig. 3-C).

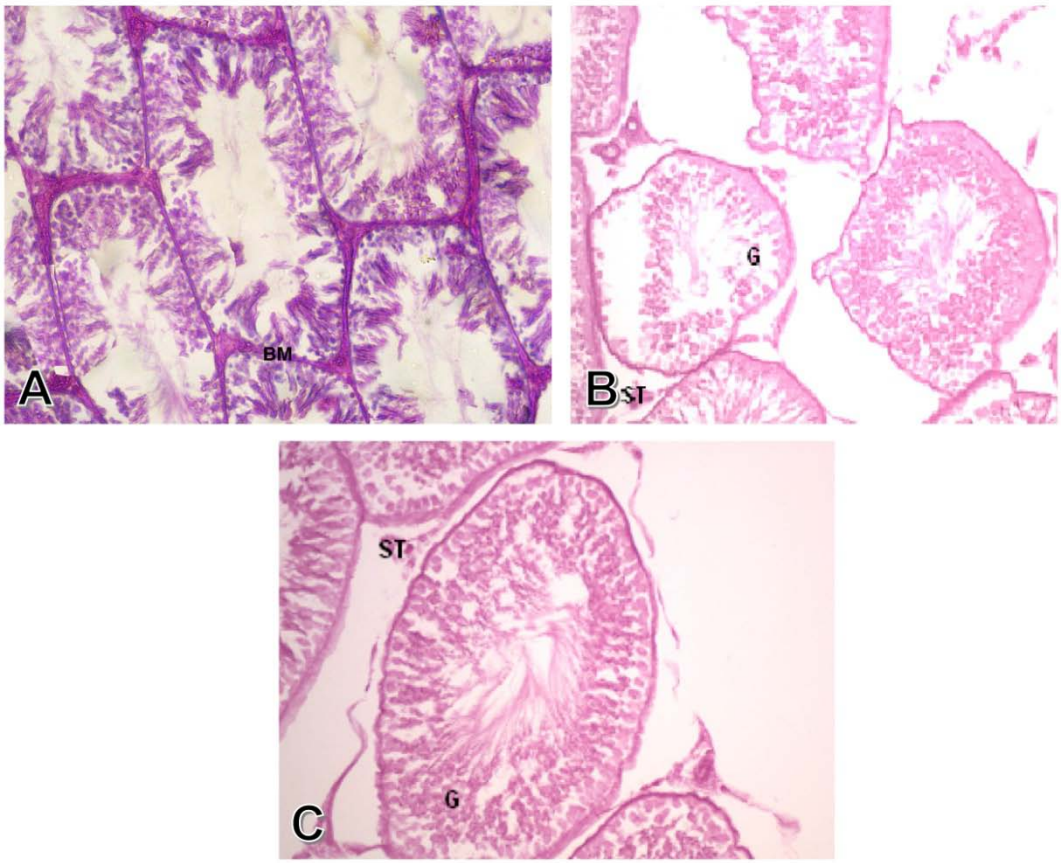

FIG. 3: (A) A photomicrograph of a transverse section of a control rat testis showing normal thickness of the basement membrane (bm) (PAS X250) (B) Photomicrograph of transverse section of testis of the treated group with alumimium chloride, showing a decreased in PAS reaction in the markedly degenerated germ cells (G) lining the seminiferous tubules , as well as decreased PAS reaction of the interstitial cells (ST), (PAS X 250) (C) Photomicrograph of transverse section of testis of the group treated with alumimium chloride, and propolis, showing marked increase in PAS reaction of the seminiferous tubules components including the germ cells (G), and also increase in PAS reaction of interstitial cells of Leydig (ST).(PAS x 250).

II Ultrastructure studies

Group I: Control group.

The spermatogonia were large diploid cells that lied against the boundary tissue of the seminiferous tubules. The A-type spermatogonia had large pale ovoid nuclei, a scantly granular cytoplasm, and rough endoplasmic reticulum, the mitochondria were spherical or ovoid, and a simple Golgi apparatus. The B-type cells had rounded nuclei, and a granular cytoplasm. Primary spermatocytes had spherical nuclei with fine granular nucleoplasm, scanty cytoplasm, and ovoid mitochondria that aggregated in groups, the Golgi apparatus arranged in few clusters accumulated at one pole of the cell. The secondary spermatocytes were rarely seen, they entered into the second meiotic division producing the spermatids. The early spermatids were rounded cells with large spherical nuclei that contained clumps of chromatin in a lightly stained cytoplasm, the endoplasmic reticulum had flattened vesicles, the mitochondria aggregated at the periphery of the plasma membrane. The cytoplasm of Sertoli cells extended from the basal lamina to the lumen of the seminiferous tubules. The nucleus was enfolded and the cytoplasm contained abundant endoplasmic reticulum, ovoid Golgi apparatus and spherical mitochondria (Figs. 4-A1 & A2).

Group II: Treated with aluminum chloride.

The seminiferous tubules, showed cellular alteration of cells than those in the control groups; the tubular membranes were dilated with irregular Sertoli cells, the spermatogonia contained vacuolated cytoplasm and irregular nuclei, the mitochondria were swollen, the Golgi apparatus contained lipid droplets. The spermatid cells were atrophied, with degenerated nuclei and defected acrosomal caps, degenerated mitochondria, and dilated endoplasmic reticulum (Figs. 4-B1 & B2).

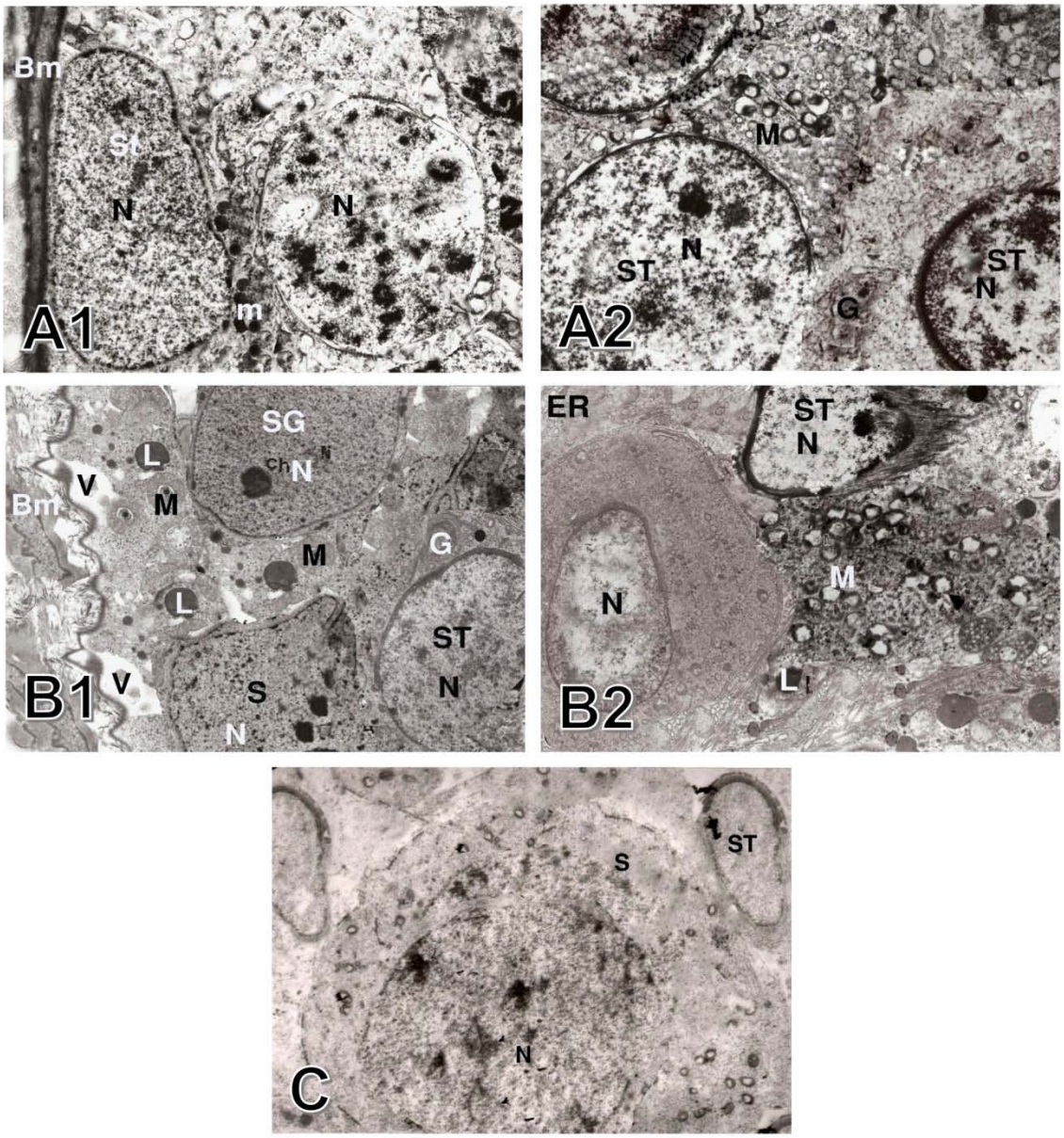

FIG 4: (A1) Photo electron micrograph of the testis of a control rat, showing basement tubular membrane (Bm) A and B types spermatogonia (St), nuclei (N), mitochondria (m) .(X120000). (A2) Photo electron micrograph of control rat testis, showing spermatid (ST) and its nucleus (N), mitochondria (M) and Golgi apparatus (G).(X12000). (B1) Photo electron micrograph of testis group treated with aluminum chloride , showing dilated irregular tubular membrane (Bm),Sertoli cell (S) , spermatogonia(SG) and early spermatid (ST), and vaculated cytoplasm (V) , irregular nuclei (N), swollen mitochondria (M), Golgi apparatus (GA), lipid (L).(X12000). (B2) Photo electron micrographs of rat testis group treated with aluminum chloride, showing degenerative and atrophy of spermatid cells (ST), degenerative nuclei(N), and defects in acrosomal cap formation, degenerated mitochondria (M), dilated endoplasmic reticulum (ER) and cytoplasmic vacuoles (V). (X18000). (C) Photo electron micrograph of rat testis, group treated with aluminum chloride and propolis showing nearly normal spermatid (ST) and secondary spermatocyte (S). (X12000).

Group III: Treated with propolis with concomitant administration of aluminum chloride.

Sections showed no histological changes in the germ cells which, lined the seminiferous tubules with normal basement membrane (Fig. 4-C).

IV. Discussion

Our studies postulated that aluminum chloride exposure caused marked alterations in the histological pattern of the tests which were represented by some of the seminiferous tubules were degenerated, while others appeared necrotic. In addition to degeneration and

necrosis of germ cells lining of the seminiferous tubules with thick basement membrane and fragments of germ cells in the center of seminiferous tubules. These results parallel to Liobet et al.[21], Guo et al.[23], Fairoze [24], Burdock [25], Yousef et al. [54], and Hala et al.[55], who founded that aluminum chloride caused histopathological lesion in testis as congestion of interstitial blood vessel, marked degeneration and necrosis of the germ cells, absence of spermatic bundles in seminiferous tubules lumen and this might result from degeneration of the spermatogonia which affected spermatogenesis and maturation of sperms, and this supported our results. The role of aluminum chloride in induction of oxidative stress, this toxic effect might result from its ability to bind to cellular DNA, RNA, and proteins, leading to a decrease of their levels in different tissues [56]. And also, the mechanisms that might explain, the degeneration, the coagulative necrosis, and the epithelial sloughing in our results [56], and [57]. According to Fraga et al. [58] and Kumar et al. [59], the presence of interstitial edema in our results might result from the oxidative damage of capillaries endothelial cells through interference with their membranes, which increased their permeability, the increase in permeability and edema might also occur in inflammatory responses due to the liberation of inflammatory mediators from the degenerated and the necrotic parenchymatous cells, and this might explain inflammatory cells infiltration within testicular parenchyma in our results. Our results showed some fibrosis of the interstitial tissue in the markedly degenerated germ cells lining the seminiferous tubules, as well as decreased PAS reaction of the interstitial cells these findings were in agreement with the results of Chinoy et al. [60] who reported profound disorders in carbohydrate metabolism, follow aluminum chloride toxicity. The degeneration and atrophy of spermatid cells, revealed in the present study agreed with the results obtained by Libet et al. [21] and Guo et al. [23]; who found that there was necrosis in the spermatocytes and spermatids after administration of aluminum nitrite to mice, and the gonadal toxicity of rats exposed to, aluminum chloride, which recorded by Alfrey [61], Krasovskii et al. [62], and Alfrey et al. [63]. In the present study, the pathological changes demonstrated in testicular ultrastructure as irregular Sertoli cell, vacuolated cytoplasm and defected spermatogonia after administration of aluminum chloride were in agreement with Khattab [22], and Kamboji and Kar [64], who reported that, the seminiferous tubules were shrunken with spermatogenic arrest at the primary spermatocytes or spermatogonia stages with the treatment by aluminum sulfate. In the present study, some pathological changes in early spermatid with vacuolated cytoplasm and irregular nuclei after administration of aluminum chloride were in agreement with the work of Mayyas, et al. [65], who reported destruction of the seminiferous tubules with large necrotic areas and degenerated cells. Our present study showed no histological changes in the germ cells which line the seminiferous tubules as well as normal interstitial cells of Leydig with lack of congestion of interstitial blood vessels in the testis of rats treated with aluminum chloride with the concomitant treatment with propolis extract, these results agreed with Hasan et al. [66], who reported that the treatment with propolis antagonized aluminum chloride toxicity on liver and testis. The antioxidant property of propolis responsible for its protective effect in our results agreed with Chang et al. [67], Gómez-Caravaca et al. [68], and Mokhtar et al. [69] who reported that, propolis antagonized the harmful reproductive toxicity of aluminum chloride on testis. Propolis reduced the oxidative stress, apoptosis, and necrosis induced by aluminum chloride on the testes seen in the results approved by El Masry et al. [70], and Hasan et al. [66]. In the present study, co-administration of propolis practically prevented most of the toxic effects of aluminum chloride on the testis, these results agreed with Alyane et al. [71] who reported that pretreatment of rats with propolis extract reduced the peroxidative damage in the mitochondria as reducing both of mitochondrial malondialdehyde formation and the amplitude of mitochondrial swelling.

V. Conclusion

Our results demonstrated that the aluminum chloride induced toxic structural changes in the rat's testicular tissues, which could be improved by the protective effect of propolis supplement. These results validate the hypothesis that the testicular toxicity of aluminum chloride modulated by propolis supplementation.

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Materials and Methods: A total of 40 male Wistar rats were randomly divided into the following five groups: control, diabetes mellitus, diabetes mellitus with vehicle treatment, diabetes mellitus with propolis treatment (100 mg/kg) and diabetes mellitus with propolis treatment (200 mg/kg). Diabetes mellitus in rats was induced by intraperitoneal injection of streptozotocin (60 mg/kg). Diabetic groups were treated with vehicle or ethanolic extracts of Iranian propolis for 6 weeks. Serum concentration of malondialdehyde, superoxide dismutase and glutathione peroxidase were measured.

EEP at dose levels of 200 mg/kg bodyweight by oral gavages, daily for 6 weeks.

Hamid Reza Sameni, Parisa Ramhormozi, Ahmad Reza Bandegi, Abbas Ali Taherian 1, Majid Mirmohammadkhani, Manouchehr Safari “Effects of ethanol extract of propolis on histopathological changes and anti-oxidant defense of kidney in a rat model for type 1 diabetes mellitus” J Diabetes Investig Vol. 7 No. 4 July 2016.

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Comparison between Different Techniques for Determining Hematological Parameters in Dogs

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Abstract- The objective was to compare the hematological parameters obtained using automated, manual and estimated evaluation techniques, in order to verify whether these techniques can be used with confidence in dogs. Samples from 297 dogs were submitted to automated, manual and estimated blood count based on the hematocrit leveus and platelet estimation. Fisher's Exact Test, sensitivity, specificity calculations, positive predictive value, negative predictive value and kappa agreement coefficient (k) ($p < 0.05$) were performed taking into account manual analysis as the gold standard. Hematocrit, hemoglobin and erythrocytes, presented ($k = 0.67; 0.67; 0.71$), and $p < 0,01$. Total leukocytes (TL), lymphocytes and platelets showed ($k = 0.54; 0.44; 0.42$), $p < 0,01$. Granulocytes, monocytes and eosinophils showed ($k = 0.34; 0.07; 0.01$). Automatic mean corpuscular volume (CMV) and mean corpuscular hemoglobin (MCHM) concentration showed $p > 0,05$.

Keywords: *canine, hematological counter, hemogram.*

GJMR-G Classification: *NLMC Code: WA 360*

Strictly as per the compliance and regulations of:

Comparison between Different Techniques for Determining Hematological Parameters in Dogs

Comparação Entre Diferentes Técnicas Para Determinação Dos Parâmetros Hematológicos Em Cães

Larissa Marchiori Sena [α], Théo Matos Arantes Moraes [σ], Lorena Silveira de Almeida [ρ], Ronaldo Eugênio de Oliveira [ω], Ana Paula Madureira [¥] & Graziela Barioni [§]

Resumo- Objetivou-se comparar os parâmetros hematológicos obtidos por meio das técnicas de avaliação automatizada, manual e estimada, a fim de verificar se essas técnicas podem ser utilizadas com confiança em cães. Amostras de 297 cães foram submetidos ao hemograma automatizado, manual e técnica estimada com base no valor do hematócrito e estimativa plaquetária. Foram realizados o Teste Exato de Fisher, cálculos de sensibilidade, especificidade, valor preditivo positivo, valor preditivo negativo e coeficiente de concordância kappa (*k*) ($p<0,05$) levando em consideração a análise manual como padrão ouro. Hematócrito, hemoglobina e hemácias, apresentaram (*k*=0,67;0,67;0,71), e $p<0,01$. Leucócitos totais (LT), Linfócitos e plaquetas demonstraram *(k*=0,54;0,44;0,42), $p<0,01$. Granulócitos, monócitos e eosinófilos apresentaram (*k*=0,34; 0,07; 0,01). O volume corpuscular médio (VCM) e a concentração de hemoglobina corpuscular media (CHCM) automáticos apresentaram $p>0,05$. A análise estimada mostrou fraca concordância e $p>0,05$. Conclui-se que hemoglobina, hematócrito e hemácias automatizados podem ser utilizados. Porém, VCM e CHCM devem ser interpretados com cautela. LT podem ser utilizados apenas em animais sem alterações hematológicas. A contagem manual de leucócitos não deve ser substituída pela contagem automatizada. A contagem plaquetária automatizada pode ser empregada desde que não haja presença de agregados plaquetários. Já o eritrograma estimado, não deve ser empregado.

Palavras Chave: caninos, contador hematológico, hemograma.

Abstract- The objective was to compare the hematological parameters obtained using automated, manual and estimated evaluation techniques, in order to verify whether these techniques can be used with confidence in dogs. Samples from 297 dogs were submitted to automated, manual and estimated blood count based on the hematocrit leveus and platelet estimation. Fisher's Exact Test, sensitivity, specificity calculations, positive predictive value, negative predictive value and kappa agreement coefficient (k) ($p<0.05$) were performed taking into account manual analysis as the gold standard. Hematocrit, hemoglobin and erythrocytes, presented (k = 0.67; 0.67; 0.71), and $p<0,01$. Total leukocytes (TL), lymphocytes and platelets showed (k = 0.54; 0.44; 0.42), $p<0,01$. Granulocytes, monocytes and eosinophils showed (k = 0.34; 0.07; 0.01). Automatic mean corpuscular volume (CMV) and mean corpuscular hemoglobin (MCHM) concentration showed $p>0,05$. The estimated analysis showed poor agreement and $p>0,05$. It is concluded that hemoglobin, hematocrit and automated red blood cells can be used. However, CMV and MCHM should be interpreted with caution. TL can be used only in animals without hematological changes. Manual leukocyte counting should not be replaced by automated counting. Automated platelet count can be used as long as there is no presence of platelet aggregates. The estimated erythrogram should not be used.

Keywords: canine, hematological counter, hemogram.

I. Introdução

O hemograma é o exame mais solicitado na rotina laboratorial devido à sua praticidade, economia e utilidade. Esse exame oferece informações que podem ser utilizadas como ferramenta pelo veterinário, para que em associação aos sinais clínicos e outros exames, sirva como aliado para o diagnóstico. Assim, o hemograma é solicitado por várias razões, seja como procedimento de triagem para avaliar a saúde do animal, na busca do diagnóstico ou prognóstico, e ainda para verificar a resposta corporal às infecções e monitoramento do progresso das doenças e tratamento [(1)]

Inicialmente o hemograma era realizado por metodologia completamente manual, que mesmo sendo mais trabalhosa, demandando tempo e profissional capacitado, ainda é considerada padrão ouro para realização deste exame, sendo este método utilizada como base para a validação de novas técnicas como os contadores hematológicos automatizados segundo o *International Council for Standardization in Haematology*[(2)].

Existem ainda, estudos que estimam os valores de hemácias, e hemoglobina por meio de cálculos, partindo do princípio que esses parâmetros são uma constante proporcional ao valor do hematócrito em animais saudáveis, sendo esse método de valia para o controle de qualidade laboratorial [(3-4)].

Com o passar do tempo, as técnicas laboratoriais foram se aprimorando, e atualmente existem disponíveis no mercado contadores hematológicos automáticos, que são capazes de

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oferecer em questão de segundos os valores do eritrograma, plaquetograma e leucograma com contagens diferenciais. Esses equipamentos também oferecem parâmetros adicionais como histogramas e índices que representam a variação de cada tipo celular, além da contagem de reticulócitos [1].

No entanto, esses analisadores automáticos foram programados para reconhecer células "normais", mas quando há números significativos de células consideradas "anormais" devido a mudanças no tamanho e na forma, potenciais problemas analíticos podem ocorrer [5].

Dessa forma, devido ao grande crescimento da demanda veterinária por exames complementares e a baixa disponibilidade de estudos utilizando contadores automatizados veterinários, o objetivo do presente estudo foi comparar os parâmetros hematológicos de cães obtidos por meio das técnicas de avaliação automatizada, manual e estimada, afim de verificar se a técnica automatizada e estimada podem ser utilizadas com confiança em cães.

II. Material e Métodos

O presente estudo foi aprovado pelo comitê de ética da Universidade Federal do Espírito Santo (CEUA-UFES) sob o número 25/2018. Foram utilizadas amostras de sangue de cães de diferentes pesos, raças e idades, atendidos na rotina clínica e cirúrgica do Hospital Veterinário da Universidade Federal do Espírito Santo. Amostras sanguíneas que apresentavam alterações como desidratação seguida de hiperproteinemia, hemólise, icterícia e lipemia, foram excluídas do presente estudo.

No laboratório, após homogeneização, as amostras foram submetidas ao hemograma automático por meio do contador automatizado veterinário (Mindray BC2800vet® Mindray Bio-Medical Electronics Co., Ltd) que utiliza a impedância como principal metodologia para contagem celular. Foram quantificados os valores de hemácias (HM), hemoglobina (HB), hematócrito (HT), contagem total de leucócitos (LT), contagem diferencial de leucócitos (LD), contagem plaquetária (PLT), VCM e CHCM.

Posteriormente foram realizados os procedimentos referentes ao hemograma manual. A contabilização de HM e LT foram realizadas em hemocitômetro [3]. O HT foi realizado pela técnica tradicional do microcapilar [6].

As dosagens de HB foram realizadas por espectrofotometria em fotômetro (BIOPLUS Bio-2000®) utilizando kit colorimétrico (Labtest®), que converte a hemoglobina em ciano-meta-hemoglobina, seguindo as recomendações do fabricante [7].

Os valores estimados foram obtidos a partir do HT, sendo: HT x 0,33 = valor de HB (3) e HT/6 = valor de HM [8].

Após quantificados os parâmetros, a determinação do VCM e concentração de hemoglobina corpuscular média CHCM para as técnicas manual e estimada, utilizando as fórmulas já preconizadas na literatura [3].

O esfregaço de sangue foi realizado como descrito por [1]. Após confecção e secagem, as lâminas foram fixadas em metanol e corados com corante panótico rápido (LB Larboclin®). A LD foi realizada em esfregaço sanguíneo como descrito por [9], em aumento de 400x. Os valores obtidos nessa contagem foram utilizados para o cálculo dos valores absolutos (mm^3) [3].

Durante a avaliação do esfregaço sanguíneo para auxílio na classificação da normalidade das amostras e observação de fatores que poderiam interferir diretamente nos resultados laboratoriais, as células da série vermelha e branca, foram avaliadas quanto a presença de alterações tóxicas, blásticas, neoplásicas, presença de policromasia, anisocitose, hipocrômica, poiquilocitose, presença de houleaux e inclusões celulares no aumento de 1000x. Todos esses parâmetros foram quantificados pelo sistema de cruzes sendo: (+) alteração leve, (++) alteração moderada, (+++) alteração intensa [1].

A PLT foi realizada pelo método de observação no esfregaço sanguíneo que mesmo sendo um método estimado, é utilizado com grande frequência na maioria dos laboratórios para validação dos resultados automatizados [10].

A presença de alterações plaquetárias como macroplaquetas e agregados plaquetários também foram avaliadas e quantificadas pelo sistema de cruzes sendo: (+) alteração leve, (++) alteração moderada, (+++) alteração intensa [1].

Todas os testes foram realizados em até 12 horas após a entrada do sangue no laboratório, sendo as amostras mantidas refrigeradas entre 4 e 8 °C. As avaliações foram realizadas sempre pelo mesmo observador um patologista clínico experiente e conferidas por um segundo avaliador. Os intervalos de normalidade para valores hematológicos foram determinados de acordo [3].

As análises estatísticas foram feitas utilizando o software estatístico GraphPad Prism 5.0® (Graph Prism Inc., San Diego, CA). Foram realizados o teste exato de Fisher para verificar associação entre os métodos, cálculos de sensibilidade especificidade, valor preditivo positivo (VPP) e valor preditivo negativo (VPN) seguidos pelos seus respectivos intervalos de confiança, considerando o teste manual como padrão ouro. Foi calculado o coeficiente de concordância kappa (k) para verificar a concordância entre os métodos avaliados, com nível de significância de 95% ($p<0,05$). Além disso, para melhor interpretação, os dados foram submetidos a ANOVA paramétrica e comparação de

médias de teste T de Student também a 95% de significância (p<0,05).

III. Resultados

Os resultados referentes a sensibilidade, especificidade, VPP, VPN seus respectivos IC, *p* valor referentes ao Teste Exato de Fisher (P<0,05) e índice de concordância Kappa, dos parâmetros HT, HM, HB, VCM e CHCM estão dispostos na Tabela 1.

Tabela 1: Valores de sensibilidade, especificidade, VPP, VPN, com seus respectivos intervalos de confiança (95%), *p* valores referentes ao Teste Exato de Fisher (p<0,05) e coeficiente de concordância Kappa (*k*) dos parâmetros: hematócrito, hemoglobina, hemácias, CHCM, VCM e plaquetas, obtidas por meio das técnicas automatizadas e estimadas, levando em consideração a metodologia manual como padrão ouro.

Parâmetro		Automatizado	Estimado
Hematócrito (%)	Sensibilidade	97% (0,92 a 0,99)	-
	Especificidade	74% (0,66 a 0,80)	
		p= <0,0001	
	VPP	71% (0,64 a 0,78)	
	VPN	97% (0,93 a 0,99)	
		k= 0,67	
Hemácias (mm³)			
	Sensibilidade	84% (0,76 a 0,90)	41% (0,32 a 0,50)
	Especificidade	84% (0,78 a 0,89)	65% (0,58 a 0,71)
		p= <0,0001	*p*= 0,26
	VPP	78% (0,69 a 0,84)	44% (0,35 a 0,54)
	VPN	88% (0,83 a 0,93)	62% (0,55 a 0,69)
		k= 0,67	*k*= 0,06
Hemoglobina (g/dL)	Sensibilidade	97% (0,94 a 0,99)	35% (0,27 a 0,43)
	Especificidade	74% (0,66 a 0,81)	53% (0,45 a 0,61)
		p= <0,0001	*p*=0,059
	VPP	78% (0,72 a 0,74)	42% (0,33 a 0,51)
	VPN	97% (0,92 a 0,99)	45% (0,38 a 0,53)
		k= 0,71	*k*= -0,11
CHGM	Sensibilidade	27% (0,19 a 0,36)	0,0% (0,0 a 0,29)
	Especificidade	85% (0,79 a 0,80)	100% (0,97 a 1,0)
		p= 0,004	*p*=1,0
	VPP	56% (0,43 a 0,69)	-
	VPN	64% (0,57 a 0,70)	-
		k= 0,14	*k*=0,0
VCM	Sensibilidade	29% (0,13 a 0,50)	0,0% (0,0 a 0,07)
	Especificidade	85% (0,80 a 0,89)	100% (0,98 a 1,0)
		p= 0,05	*p*= 1,0
	VPP	17% (0,07 a 0,30)	-
	VPN	92% (0,88 a 0,95)	-
		k=0,11	*k*=0,0

Sendo: Volume corpuscular médio (VCM) e Concentração de hemoglobina corpuscular média (CHCM). Valor preditivo positivo (VPP), valor preditivo negativo (VPN).

Dentre as amostras avaliadas, 40,4% (n=120) foram considerados anêmicos com base na diminuição de um ou mais parâmetros hematológicos (HM, HT e HB). O HT esteve baixo em 100% (n=120) dos casos de anemia, com médias e desvios padrões de 34,8±9,5 e 37,9±9,3 (Tabela 2) para a técnica automatizada e microcapilar respectivamente. Esses dados apontam para uma subestimação dos valores do HT obtidos pela técnica automatizada o que levaria a diminuição dos valores de especificidade e VPP observados neste estudo.

Tabela 2: Médias e desvios padrões dos parâmetros hemácias, hemoglobina, hematócrito, plaquetas, VCM, CHCM, leucócitos totais, granulócitos, linfócitos, eosinófilos, basófilos e monócitos de amostras de sangue de 297 cães, obtidos por meio das técnicas automatizada, manual e estimada.

Parâmetro	Manual	Automatizado	Estimado
Hematócrito (%)	37,91±9,51[a]	34,87±9,35[b]	-
Hemoglobina (g/dL)	11,52±3,21[a]	10,81±3,08[b]	11,37±3,37[c]
Hemácias (mm)³	7,03±6,37[ac]	5,76±2,89[b]	6,25±1,35[bc]
CHGM	30,19±2,80[a]	31,18±4,32[a]	33,00±0,0[b]
VCM	68,16±5,01[a]	62,90± 6,37[b]	60,00±0,0[c]
Leucócitos totais / mm³	14479±12042[a]	12612±9465[b]	-
Linfócitos / mm³	20,12±12,35[a]	21,23±10,88[a]	-
Granulócitos / mm³	71,91±15,09[a]	70,50±12,21[a]	-
Monócitos / mm³	2,71±3,00[a]	4,88±1,50[b]	-
Eosinófilos / mm³	5,23±3,59[a]	3,44±9,64[b]	-
Plaquetas /μL	-	310,94±181,01[a]	469,13±892,35[b]

Os valores plaquetários são referentes a n x 10³.

Entretanto, o valor de *k* foi considerado satisfatório mostrando que o HT pode ser obtido por meio desta técnica em amostras sanguíneas de cães. Porém em 26% dos casos, esta análise não é capaz de fornecer o hematócrito real do animal, quando ele encontrava- se dentro da normalidade, levando a erros diagnósticos.

A mensuração da HM pelo contador automatizado apresentou valores de sensibilidade e especificidade idênticos (84%), além de boa correlação entre os métodos. Entretanto, a análise estimada mostrou baixa sensibilidade e especificidade, além de fraca correlação com a metodologia manual, de maneira que esse método não deve ser utilizado na rotina clínica.

A HB mensurada pelo analisador automático apresentou excelente índice de correlação com a análise pela espectrofotometria (k= 0,71). Já a técnica estimada mostrou-se insatisfatória com baixa sensibilidade, especificidade e concordância quase nula entre os testes.

É interessante ressaltar a baixa sensibilidade e fraca concordância encontrada nos índices hematimétricos CHCM e VCM obtidos por meio da análise automatizada, levando a erros interpretativos graves que podem influenciar na conduta clínica do veterinário.

O leucograma automatizado obteve resultados menos satisfatórios quando comparados ao eritrograma. Os valores referentes a sensibilidade, especificidade, VPP, VPN seus respectivos IC, *p* valor referentes ao Teste Exato de Fisher (P<0,05) e índice de concordância Kappa, dos parâmetros LT, LD e PLT estão dispostos na tabela 3.

Tabela 3: Valores de sensibilidade, especificidade, VPP, VPN, com seus respectivos intervalos de confiança (95%), *p* valores referentes ao Teste Exato de Fisher (p<0,05) e coeficiente de concordância Kappa (*k*) dos parâmetros: leucócitos totais, granulócitos, eosinófilos, linfócitos e monócitos de amostras sanguíneas de cães, obtidas por meio das técnicas automatizadas e estimadas, levando em consideração a metodologia manual como padrão ouro.

Parâmetro		Automatizado	Estimado
Leucócitos totais/ mm³	Sensibilidade	70% (0,60 a 0,79)	-
	Especificidade	84% (0,78 a 0,89)	
		p= <0,0001	
	VPP	66% (0,55 a 75%)	
	VPN	87% (0,81 a 0,91)	
		k=0,54	
Granulócitos / mm³	Sensibilidade	58% (0,49 a 0,66)	-
	Especificidade	75% (0,68 a 0,82)	

		p= <0,0001	
	VPP	67% (0,58 a 0,75)	
	VPN	67% (00,60 a 0,74)	
		k=0,34	
Eosinófilos / mm³	Sensibilidade	52% (0,43 a 0,60)	-
	Especificidade	49% (0,41 a 0,58)	
		p= 0,20	
	VPP	49% (0,41 a 0,58)	
	VPN	57% (0,49 a 0,65)	
		k=0,07	
Monócitos / mm³	Sensibilidade	8% (0,04 a 0,13)	-
	Especificidade	93% (0,85 a 0,96)	
		p= 0,65	
	VPP	52% (0,29 a 0,74)	
	VPN	54% (0,48 a 0,60)	
		k= 0,01	
Plaquetas /µL	Sensibilidade	69% (0,58 a 0,78)	-
	Especificidade	76% (0,69 a 0,81)	
		p= <0,0001	
	VPP	56% (0,46 a 0,65)	
	VPN	84% (0,78 a 0,89)	
		k=0,42	

Os valores de granulócitos são referentes a soma de neutrófilos segmentados, bastões e basófilos. Sendo: Valor preditivo positivo (VPP), valor preditivo negativo (VPN). Na avaliação plaquetária o método estimado foi considerado como padrão ouro para a análise.

LT, linfócitos e granulócitos apresentaram os melhores resultados com (p<0,001) mostrando a associação entre os métodos, porém com concordância kappa considerada moderada para os dois primeiros parâmetros e fraca para os granulócitos. Desvio nuclear de neutrófilos a esquerda foram observados 7,1 % das amostras (n=21), e estes não puderam ser identificado pela análise automatizada.

A contagem total de leucócitos automatizada apresentou 198 valores de especificidade (84%) e VPN (87%) considerados satisfatórios, mostrando que essa leitura deve ser empregada com confiança apenas em animais com leucograma dentro da normalidade.

Na análise plaquetária, o método automático apresentou melhor capacidade de detector animais saudáveis com 76% de especificidade, índice de concordância moderado e (p<0,05). Porém, VPP que neste caso demonstra a probabilidade de um paciente classificado como trombocitopênico ou com trombocitose realmente apresentar essas alterações foi de apenas 56%.

IV. Discussões

Para a determinação do HT pela impedância, primeiramente é determinado o VCM por meio de um histograma de distribuição e a partir deste dado é determinada o percentual total de glóbulos vermelhos no volume injetado determinando o HT [11]. Dessa forma quaisquer fatores que influenciarem a determinação do VCM alterarão o HT.

Amostras sanguíneas que apresentem anisocitose e poiquilocitose acentuada, além da presença de macroplaquetas, podem interferir na correta determinação do tamanho e estimativa celular, alterando a mensuração do VCM e consequente o HT [12]. Porém, uma pequena parcela dos animais, apresentaram as alterações descritas acima, explicando os bons resultados na determinação de animais realmente anêmicos.

A quantificação de HM realizada pelo equipamento automatizado é feita pelo método de impedância criado por Coulter. Este método baseia-se na medição das alterações da resistência elétrica produzida por uma partícula, de forma que a amplitude de cada pulso é proporcional ao volume de cada partícula [13]. Assim, a quantificação das HM é uma análise direta, não sendo secundária a outros fatores como o hematócrito, o que determina os bons resultados obtidos por essa técnica.

Baseando-se no fato da metodologia para determinação da HB em ambos as técnicas serem por conversão da HB em ciano-meta-hemoglobina [6]. Era esperado a boa correlação entre as metodologias, demonstrando que a metodologia automática pode ser

empregada com segurança para mensuração deste parâmetro.

Trabalhos que avaliaram a relação hemoglobina: hematócrito em bovinos, observaram a subestimação dos valores de HB quando determinadas por um terço do HT, determinando uma nova constante para o cálculo de HB na espécie [4].

A existência da constante determinando que o valor da HB constitui um terço do HT parte do princípio de que todas as HM carregam concentrações ideais de HB, dessa forma, explica-se os resultados insatisfatórios e a necessidade de novos trabalhos determinando equações para a quantificação da HB em cães.

Se tratando do VCM, a ausência de concordância entre as técnicas pode ser explicada pela diferença na metodologia para determinação desses índices. No caso da análise manual, calcula-se HM, HT e a partir de uma fórmula determina-se o VCM. Já a técnica automatizada primeiro calcula-se o VCM e a partir deste determina-se o HT. Dessa forma, a influência do fator hematócrito na análise manual, pode ter sido determinante para os resultados obtidos. Vale ressaltar ainda, que resultados insatisfatórios na determinação do VCM explicam erros dos valores de HT obtidos pela impedância.

O alto VPN (92%) do VCM na análise automatizada demonstra a alta probabilidade de animais com VCM dentro da normalidade realmente estarem normais, salientando a necessidade de maior atenção principalmente em amostras com este índice hematimétricos fora da normalidade.

Já o CHCM é calculado da mesma maneira em ambas as técnicas, dessa forma, as alterações no HT podem ter sido responsáveis pelas alterações no CHCM, levando em consideração a excelente concordância na determinação da HB.

Na análise estimada os valores do VCM e CHCM foram insatisfatórios por terem sido calculados com HM e HB também estimadas e que não apresentaram correlação com a análise manual, por serem como uma constante do HT. Assim, essa análise sempre classifica os animais com anemias normocíticas normocrômicas, explicando a sensibilidade de 0,0% e especificidade de 100%.

Discordâncias entre a contagem total e diferencial de leucócitos são decorrentes dos princípios para determinação celular que esses aparelhos apresentam. A contagem de leucócitos totais é realizada pela passagem celular na zona de detecção semelhante as hemácias, de maneira que uma substância que lisa as hemácias é aplicada e são quantificadas todas as células nucleadas. Porém, a contagem diferencial é feita com cálculos por meio do % do tamanho das células que passaram pela zona de detecção [6].

Assim, os analisadores automáticos foram programados para reconhecer células de tamanho e formato normais, dessa forma, quando há números significativos de células considerada fora do padrão, como em casos de neoplasias, neutrófilos tóxicos, monócitos reativos, podem ocorrer problemas de diferenciação e consequentemente quantificação cellular [5].

Corroborando a estes dados, variações de 0 a 37% foram obtidas na contagem diferencial leucocitária entre diferentes metodologias de contagem automatizada [14]. Além disso, outros trabalhos descrevem que fatores como macroplaquetas e hemácias nucleadas poderiam interferir nos resultados. Ao utilizar a metodologia automatizada para realização do hemograma, valores errôneos são obtidos em relação a leucometria total e diferencial na presença de eritrócitos jovens e macroplaquetas [15].

No entanto metarrubrícitos e células blásticas foram observados em quantidades significativas em uma pequena parcela amostral, demonstrando que outros fatores podem estar envolvidos nos baixos resultados da contagem diferencial liberada pelo contador hematológico.

A não diferenciação de bastonetes no contador avaliado também é um fator crucial que diminui a eficiência desse equipamento, pois impossibilita o diagnóstico de desvio de neutrófilos a esquerda, mascarando casos de infecção, tornando indispensável a avaliação da lâmina de esfregaço sanguíneo pelo patologista clínico.

Discordâncias plaquetárias são decorrentes provavelmente da presença de agregados plaquetários que se formam durante a coleta e observados na leitura do esfregaço sanguíneo [16]. Esses artefatos também foram capazes de comprometer a eficácia da determinação plaquetária em lâmina durante o presente estudo, podendo ter interferido nos resultados.

Estudos que compararam as técnicas de estimativa plaquetária em esfregaço sanguine com a contagem automática pela metodologia óptica, contagem plaquetária pela impedância e a avaliação imunológica, forneceram evidências de que determinação estimada, metodologia óptica e a técnica imunológica são superiores aos contadores automáticos que utilizam métodos de impedância [17]. Outras pesquisas, também forneceram evidências que em animais com anemias microcíticas acentuadas o método de impedância superestimou significativamente as contagens de plaquetas de forma a hemácias de pequeno tamanho podem ser confundidas com plaquetas [10].

V. Conclusões

Os parâmetros hemácias, hemoglobina e hematócrito obtidos por meio da análise automatizada

podem ser utilizados na rotina clínica. No entanto, VCM e CHCM devem ser interpretados com cautela. A contagem total de leucócitos pode ser empregada apenas quando não há alterações hematológicas na amostra, sendo provenientes de animais hematologicamente sadios. No entanto, inclusive nessas circunstâncias a contagem diferencial de leucócitos deve ser realizada em lâmina pelo patologista clínico. A contagem plaquetária automática pode ser empregada desde que não haja presença de agregados plaquetários. O eritrograma estimado com base no valor do hematócrito não pode ser utilizado em amostras sanguíneas de cães.

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Antivirt ® $\{Al_4\ (Sio_4)_3 + 3Mg_2SiO_4 \rightarrow 2Al_2\ Mg_3\ (Sio_4)_3\}$ Enhances Efficacy of Co-trimoxazole to Terminate Experimental Trypanosome-Infections in Mice

By M. C. O. Ezeibe, M. I. Ezeja, C. A, Akpan, F. I.O. Onyeachonam, M. E. Sanda, I. J. Ogbonna, E. Kalu, N.U. Njoku & M.I. Udobi

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Abstract- Both bacteria and protozoa require Folic acid for replication and Cotrimoxazole inhibits synthesis of the vitamin. For its mechanism of inhibiting Folic acid, the medicine has been in use as antibacterial drug for many decades but it is not being used to treat trypanosomosis (protozoan disease). To enhance anti-Folic acid activity of the medicine in order to improve its anti-trypanosome efficacy and make it function as new medicine for sleeping sickness (tropical disease of man and animals) it was stabilized with Antivirt® (Medicinal synthetic Aluminum-magnesium silicate). At 100 % of its antibacterial dose, Cotrimoxazole significantly reduced ($P \leq 0.05$) trypanosome parasitemia in mice, from 12.76±1.20 to 5. 87± 0.43. When it was stabilized with the Antivirt®, 75 % of the antibacterial dose had slight reduction ($P \geq 0.05$) in the trypanosome parasitemia (11.30±1.01) while the 100 %-dose achieved zero (0.00±00) trypanosome parasitemia and improved total WBC counts (immunity) from 1.50±0.16 to 2.86±0.38.

Keywords: cotrimoxazole; folic acid-inhibition; anti-trypanosomes efficacy; antivirt®; sleeping sickness, new medicine.

GJMR-G Classification: NLMC Code: QW 70

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M. C. O. Ezeibe [α], M. I. Ezeja [σ], C. A, Akpan [ρ], F. I.O. Onyeachonam [ω], M. E. Sanda [¥], I. J. Ogbonna [§], E. Kalu [χ], N.U. Njoku [ν] & M.I. Udobi [Θ]

Abstract- Both bacteria and protozoa require Folic acid for replication and Cotrimoxazole inhibits synthesis of the vitamin. For its mechanism of inhibiting Folic acid, the medicine has been in use as antibacterial drug for many decades but it is not being used to treat trypanosomosis (protozoan disease). To enhance anti-Folic acid activity of the medicine in order to improve its anti-trypanosome efficacy and make it function as new medicine for sleeping sickness (tropical disease of man and animals) it was stabilized with Antivirt® (Medicinal synthetic Aluminum-magnesium silicate). At 100 % of its antibacterial dose, Cotrimoxazole significantly reduced (P≤ 0.05) trypanosome parasitemia in mice, from 12.76±1.20 to 5. 87± 0.43. When it was stabilized with the Antivirt®, 75 % of the antibacterial dose had slight reduction (P≥ 0.05) in the trypanosome parasitemia (11.30±1.01) while the 100 %-dose achieved zero (0.00±00) trypanosome parasitemia and improved total WBC counts (immunity) from 1.50±0.16 to 2.86±0.38.

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I. Introduction

Trypanosomosis is debilitating and often fatal in both man and animals [1] [2]. The disease is found mainly in sub-Saharan Africa [3]. It has wide range of animal-hosts [4] [5] and is transmitted by *Glossina spp*, flies (*Tabanids* and *Stomoxys*) and Vampire bats. Dourine (caused by *T. equiperdum*) is sexually transmitted in Equines.

African trypanosomosis has been reported to be responsible for 55,000 human and 3 million livestock deaths, annually [6]. In Nigeria, *Trypanosoma brucei* and *T. congolense* are the most pathogenic species for domestic animals [7].

The disease is a major cause of mortality in animals in Nigeria and contributes greatly to under-development of Sub-Saharan Africa and other socio-economic consequences despite huge amounts being spent on research to control it [8]. World health organization [9] reported that over 60 million people and 48 million livestock in Africa, are at risk of \trypanosomosis. Estimated losses due to trypanosomosis in Africa run into billions of Dollars.

Of the different species of Trypanosomes which affect man and animals, *Trypanosoma. congolense* is strictly a parasite of the microcirculation, producing primary lesions in blood vessels and lymph nodes [10] [11] [12]. *Trypanosoma brucei* is found in connective tissues, producing inflammation, cellular degeneration and necrosis which lead to tissue and organ damage [4] [12].

Control measures for trypanosomosis are either by controlling the vectors or by use of chemotherapy or a combination of both. In poor, rural communities, affected by the disease, control is mainly by use of trypanocidal drugs [13]. Drugs currently employed in treatment of trypanosomosis are: Homidium salts (Ethidium-Novidium®:); Quinapyramine sulfate (Antrycide®:); Diminazene aceturate (Berenil®:); Isometamidium (Samorin-Trypamidium®:) and Suramin sodium. These drugs have been in use for more than half a century now [14]. It is estimated that 35 million doses of the drugs are used in Africa each year, with about 50–70 million animals at risk [14].

Microorganisms exposed to drugs over such a long time usually develop resistance to the drugs. Mechanisms for drug-resistance include, loss of surface specific receptors or transporters for the drugs, increased metabolism of the drugs and alteration (by mutation) of specific targets for the drugs on the organisms. These result in resistance to a small number of related drugs, too. More often, cells express mechanisms of resistance that confer simultaneous resistance to many different structurally and functionally unrelated drugs [15]. For the problem of drug-resistance by pathogens, there is need for constant search for new drugs for treatment of important diseases such as trypanosomosis. Cotrimoxazole is a combination of five parts of sulfamethoxazole and one part of trimethoprim based on dose of each of the two drugs. It is being used for treatment of bacterial, fungal and protozoan

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infections [16]. The drug-formulation was introduced into clinical use in the late 1960s and it is for treatment of urinary tract infections, respiratory infections, sexually transmitted diseases, enteric infections and typhoid fever. Advantages of Cotrimoxazole include combination of the two components. The drug inhibits synthesis of tetrahydrofolic acid which is needed as a cofactor in synthesis of thymidine and purines which are components of bacterial DNA [17] [18] [19]. Sulfamethoxazole inhibits synthesis of the intermediary dihydrofolic acid from its precursors [20] while Trimethoprim competitively inhibits dihydrofolate reductase and consequently, production of tetrahydrofolic acid from dihydrofolic acid [21]. Potentiating sulfamethoxazole with trimethopriim reduces toxicity and microbial resistance [22]. The synergy between trimethoprim and sulfamethoxazole was first described in the late 1960s [23]. Trimethoprim and sulfamethoxazole have a greater effect when given together than when given separately, because they inhibit successive stages in the folate synthesis pathway. They are formulated in a ratio of one-to-five so that when they enter the body their concentrations in the blood and tissues are exact ratio required for a peak synergistic effect between the two [24]. Trimethoprim causes a backlog of dihydrofolate and this backlog can work against inhibitory effect the drug has on tetrahydrofolate biosynthesis. This is where the sulfamethoxazole comes in. Its role is in depleting the excess dihydrofolate by preventing it from being synthesized in the first place.

Trimethoprim-sulfamethoxazole (Cotrimoxazole) has proved effective in the treatment of infections of coccidian protozoa parasites, Isospora and Cyclospora [25] [26]. So, by potentiating its anti-folic acid effects its protozoan efficacy may be enhanced to achieve cure for trypanosmosis.

Molecules of Aluminum magnesium silicate (AMS: clay) are 0.96 nanometer thick and some hundred Nanometers across [27] [28] [29]. As a Nanomedicine, AMS helps in delivering drug-molecules to target cells. Drug molecules in “corridors” of AMS “house of cards” are also bound by charged faces and edges of its platelets. So, they are protected from degradation by both physical and physiological factors but are released gradually into blood of treated patients. Also, silicates are immune stimulants [30] and AMS is a stabilizing/potentiating agent [31] [32]. By stabilizing drugs, AMS increases potency of the drugs [33]. When drugs are potentiated their doses required for desired effects reduce and using lower doses for treatments reduces side effects of drugs so that immune responses of patients improve. With enhanced efficacy of drugs and improved immune responses of patients both sensitive infections and drug-resistant infections could be effectively treated. So, using The Medicinal synthetic Aluminum-magnesium silicate (Antivirt®) to stabilize Cotrimoxazole may enhance its efficacy against trypanosomes enough so that the medicine being commonly used for treatment of bacterial diseases and amebiasis (protozoan disease) can also function as a medicine for trypanosomosis (sleeping sickness).

II. Materials and Methods

Twenty-five mice were assigned to five (5) groups of five (5) each, as follows:

Group 1: Infected/Untreated

Group 2: Infected/Treated with 100%-dose of Cotrimoxazole

Group 3: Infected/Treated with 75% -dose of Cotrimoxazole

Group 4: Infected/Treated with 100% -dose of Cotrimoxazole in Antivirt®

Group 5: Infected/Treated with 75%-dose of Cotrimoxazole in Antivirt®

Blood samples were collected from each of the mice and examined daily until parasitemia was established in all infected groups. Treatment was started 7 days post-infection and lasted for 5 days while assessment of parasitemia was on day-2 post-treatment.

a) Data analysis

The parasitemia and total WBC were presented as means ± SEM and analyzed for statistical differences by one way analysis of variance and the significant differences were accepted at the level of $P \leq 0.05$.

III. Results

Parasitemia was observed in all the groups from four days post infection and it increased steadily until treatment was commenced by day-7 post infection. Zero mean parasitaemia (0.00±00) of the group of trypanosome-infected mice treated at 100 % dose of Cotrimoxazole with Cotrimoxazole- Antivirt® drug formulation was significantly ($P \leq 0.05$) less than 5.87±0.43 of the group treated with 100 % dose of Cotrimoxazole with Cotrimoxazole alone. Both the 0.00±00 parasitaemia of the 100 % -dose of Cotromoxazole in Antivirt® group and 5.87±0.43 of the 100 % -dose of Cotrimoxazole-group were significantly ($P < 0.05$) lower than 11.73±0.86 of the 75 % -dose of Cotrimoxazole-group and 11.30±1.01 of the 75 % -dose of Cotrimoxazole in Antivirt®-group but there was no significant difference ($P > 0.05$) in mean parasitemia (11.73±0.86) of the group of 75 % -dose of Cotrimoxazole and 11.30±1.01 of the 75 %-dose of Cotrimoxazole in Antivirt®-group. Mean parasitemia of the trypanosome-infected groups of mice treated with Cotrimoxazole and Cotrimoxazole- Antivirt® are as shown on Table 1.

Mean WBC was highest in the group infected and treated with 100 %-dose of Cotrimoxazole in Antivirt® when compared with the other infected groups. The mean WBC of the group of infected/treated with 100 %-dose of Cotrimoxazole was comparable to that of the group of infected/untreated, that of the group of infected/treated with 75 %-dose of Cotrimoxazole and that of the group of infected/treated with 75 %-dose of Cotrimoxazole in Antivirt® (Table 2).

Table 1: Parasitaemia (x106/L) in Trypanosome-infected mice treated with different doses of Cotrimoxazole and Cotrimoxazole in Antivirt®

S/N	Infected/Untreated	Infected/Treated			
		Cotri		Cotri-MSAMS	
		100%	75%	100%	75%
1	15.85	6.31	12.59	0.00	12.59
2	12.59	5.01	10.00	0.00	10.00
3	12.59	6.30	12.59	0.00	12.59
4	10.00		10.00		10.00
MEAN±SE	12.76±1.20^{c}	5.87±0.43^{b}	11.73±0.86^{c}	0.00±0.00^{a}	11.30±1.01^{c}

Table 2: Total WBC (X103/µL) of Trypanosome-infected mice treated with different doses of Cotrimoxazole and Cotrimoxazole in Antivirt®

S/N	Infected/Untreated	Infected/Treated			
		Cotri		Cotri-MSAMS	
		100%	75%	100%	75%
1	1.50	1.30	1.50	2.90	1.28
2	1.39	1.56	1.77	2.78	1.56
3	1.94	2.01	1.89	2.89	1.39
4	1.17		10.00		1.00
MEAN±SE	1.50±0.16^{b}	1.62±0.21b	1.72±0.11^{ab}	2.86±0.38a	1.31±0

IV. Discussion

Significant (P≤ 0.05) reduction of parasitemia from 12.76±1.20 to 5.87±0.43 (54 % infection-reduction) in trypanosome-infected mice treated with 100 % antibacterial-dose of Cotrimoxazole is evidence that the drug has anti-trypa mosomal effect. Cotrimoxazole is known to inhibit synthesis of Folic acid and trypanosomes need the vitamin for replication. However, 54 % infection clearance is much lower than the 95 % required for treatments to be effective. This failure to achieve enough level of clearance to terminate trypanosome infections may be reason Cotrimoxazole is not yet being recommended for treatment of trypanosomosis.

When the drug was stabilized with Antivirt®, the 100 %-dose completely and significantly (P≤0.05) cleared the parasitemia (00.00±0.00). AMS is a stabilizing agent and a Nanomedicine. As stabilizing agent it prolongs time medicines remain at high concentration in blood of treated animals while as a Nanomedicine, it enhances delivery of drugs to targets. Both prolongation of time of high bioavailability of drugs and delivering them to effect-targets enhance efficacy of drugs. So, the Antivirt® may have enhanced ability of Cotrimoxazole to inhibit synthesis of Folic acid and so terminated the trypanosome infections.

As a silicate AMS also enhances immune response. So, the increase in WBC count of the mice suggests that the Antivirt® may, in addition to improving efficacy of Cotrimoxazole, have enhanced immune response of the mice. Synergy of the enhanced immune response of patients and enhanced efficacy of medicines may be responsible for the zero parasitemia achieved.

In earlier studies of effects of the Antivirt® on antimicrobial drugs, it made 75 %-dose more effective than recommended (100%) doses of drugs but in this study its effects on 100 %-dose of Cotrimoxazole were the best.

Dose used as 100 % in this study is the dose recommended for treatment of bacterial infections (not for trypanosomosis). That the 100 %-dose of Cotrimoxazole without the Antivirt® was able to reduce the trypanosome infection, significantly, suggests that if the dose is increased it may lead to cure of the infection

even without the Antivirt®. Failure to determine correct dose of Cotrimoxazole for treatment of trypanosomosis may be reason the drug has not been recommended for treatment of the zoonotic/tropical disease. That dose, used as 100 % may be 75 % of dose of the drug needed for treatment of trypanosomosis in absence of the Antivirt®. However, reducing doses by stabilizing drugs with the Antivirt® reduces side effects to enhance immune responses and also reduces cost of drug formulations.

Trypanosomosis is a very serious disease of both man and animals in the tropics and the causative agents, very often, develop resistance against existing drugs. So, there is need to constantly research for new drugs. Since the Antivirt® made Cotrimoxazole achieve total clearance of the trypanosome-infections it may cure the disease and may also make development of resistance against the new therapy difficult by achieving total clearance of infections.

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Evaluation of Active Edible Films with Nano Emulsions of Essential Oils for Minced Chicken Meat

By Naga Mallika E, Haritha M, Eswara Rao B, Srinivasa Rao T & Reddy GVB

Abstract- In the current swot, an endeavour was made to assess the physical, mechanical, microbial and antioxidant properties of alginate based edible films integrated with nanoemulsions of essential oils. Distinctive alginate films were created. Among every one of these films one best film was chosen along with control to wrap the chicken patties and adequacy of these films to broaden the timeframe of realistic usability of chicken patties during 21 days of refrigerated stocking period was assessed.

The mean thickness, grammature, opacity values, tensile strength and percent elongation at break were altogether ($P<0.05$) higher in cardamom oil nanoemulsion incorporated films contrasted with films with gingeroil nanoemulsion and control. The water Vapour permeability estimates were higher in films with ginger essential oil nanoemulsion contrasted with films of cardamom essential oil nanoemulsion. The water sorption kinetics estimations of control films were essentially ($P<0.05$) higher than the remainder of details.

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The mean Antioxidant value of ginger essential oil nanoemulsion and cardamom basic oil nanoemulsion films were 80.37 and 98.06 and cardamom basic oil nanoemulsion films were essentially ($P<0.05$) higher in their antioxidant competence than ginger essential oil nanoemulsion and control films. The mean log decrease of both E.coli and S.aureus were 1.66, 2.87 and 1.54, 2.91 for Ginger and cardamom oil added films correspondingly. The log decrease was critical for both E.coli and S.aureus yet high with S.aureus. Contingent upon the investigation of above quality parameters, film with 100μl of CEON (Cardamom Essential Oil Nano Emulsion) was chosen as best film and used to assess it's capability to expand the shelf life of chicken patties. The outcomes showed that cardamom essential oil nanoemulsion can be effectively incorporated into sodium alginate films and can successfully be utilized as a food wrap and as a palatable packaging for meat and meat items.

I. Introduction

The quality and safety of meat is profoundly subjected to the applied packaging materials. Essentially fresh and handled meat products are being packaged for averting ruining and to postpone their deterioration. Packaging additionally assists in diminishing the weight loss also which is imperative in terms of economics. (Fani et al. 2018). The current methods of packaging range from an over-wrap packaging and vacuum packaging to Modified Atmospheric packaging. As of late, a progression of new packaging advancements and materials has been created including Active packaging, intelligent packaging, edible coatings or films.

Expanding enthusiasm towards utilization of edible coatings and films was escalated in the ongoing past. Taking into account of their ecological agreeable nature, a few biopolymers, for example, starch, cellulose, chitosan, gums, alginate and protein (kheziran 2018, Jridi 2018, Romanvi 2017, Mallika 2018) can be utilized as base materials for producing edible films, as these materials offer chance of acquiring films with added advantage of viable addition of essential oils, so as to create self-motivated active packaging films. These films can perhaps assure food quality and safety, stretch the shelf life of food, decrease environmental effect on food and can expand appeal of the packaged item. But, just a set number of innovations are appropriate to meat and meat items.

Alginate is a characteristic polysaccharide extracted from the cell walls of brown sea weed and could be utilized in food industry, and is known for its biodegradability, non-harmfulness, biocompatibility, low cost and extraordinary colloidal properties which incorporate thickening, stabilizing, suspending film forming, gel creating and emulsion settling properties. Alginate films forces an incredible barrier property to oxygen and carbon dioxide, great mechanical properties and it could likewise be a decent transporter of various added substances (Kafrani et al 2016).

Essential Oils are the common enemies of oxidants and when joined straightforwardly in to the food item may adjust the taste. To stretch out the time span of usability of the food product and to shield the item from oxidation and deterioration, Essential oils can be effectively included into the edible films. They could be gradually delivered on to the food surface from the film and can stay in adequate fixation, for lengthening the shelf life of the food.

The essential oils of ginger have been accounted for their solid antimicrobial, antifungal and cancer prevention performance. (Singh et al., 2008; Noori et al. 2018). The green cardamom (Elettaria cardamomum) is local to South Asia and its basic oil,

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have curative advantages like antibacterial, antifungal, anticancerous, antispasmodic, gastroprotective and anti inflammatory activity (Mejdi et al.,2016). Essential Oils when combined directly into the food may change the organoleptic properties of the food besides causing toxicity at high concentrations or they may lose their activity while responding with environmental variables such as cooking and addition of other food ingredients. To overcome these issues, a new approach is to encapsulate active compounds and to enhance their transport by developing of nanoemulsions. Nano emulsions are colloidal scatterings framed by the blend of two immiscible stages and balanced out by a surfactant, with oil droplets of size in the scope of 20-200nms. They are optically transparent in contrast with customary emulsions and this feature is a bit of advantage for food application.

II. Materials and Methods

With this in the present investigation, an attempt was made to develop sodium alginate based edible films incorporated with natural essential oil nanoemulsions i.e., ginger and cardamom and to evaluate their effect on the quality of chicken patties. Based on the results obtained, best film was selected and the film was applied to the product and the shelf-life of the product was studied under refrigeration (4±1°C) temperature at regular intervals to record the effect of nanoemulsions of essential oil loaded sodium alginate films on quality of chicken patties and to record the efficacy of the film as an active packaging.

The experiment was conducted in two parts. Ginger and cardamom essential oils were made into nanoemulsions and they were incorporated in to sodium alginate based films to prepare active packaging films. Their quality was tested and after analysing their activity during experiment two, the best film from the above experiment was applied on to chicken meat patties and quality of chicken patties was studied in order to evaluate the efficacy of the films as active packaging films.

a) Preparation of Essential Oil Nanoemulsions

Ginger essential oil (GEO) and cardamom essential oil (CEO) each at 5 per cent v/v were selected for using in sodium alginate based films to produce active packaging films. Coarse emulsions of above essential oils were formed by continuous stirring and tween 80 was added at 1.5 percent level as surfactant. The formed coarse emulsion were ultrasonicated (Qsonica, Q500, USA) at 20 KHZ, 200 watts with 20mm diameter probe for 5minutes. The temperature of the process was controlled at less than 10^0C until formation of nanoemulsions of ginger essential oil (GEON) and cardamom essential oil (CEON).

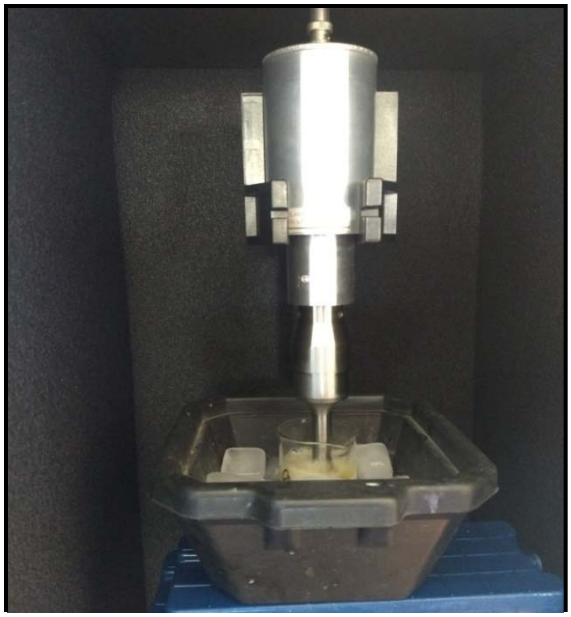
b) Preparation of Film Forming Solutions

Film forming solutions were prepared with 2% sodium alginate. Glycerol at 4 per cent level was added as plasticizer. After the temperature reached to 37^0C GEON at 10, 20 and 50 μl and CEON at 10,50 and 100 μl were added to the alginate solution to produce six different film forming solutions i.e., Sodium alginate film forming solution with 10 μl of GEON (S_1), with 20 μl of GEON (S_2), with 50 μl of GEON (S_3), with 10 μl of CEON (S_4), with 50 μl of CEON (S_5), with 100μl of CEON (S_6).

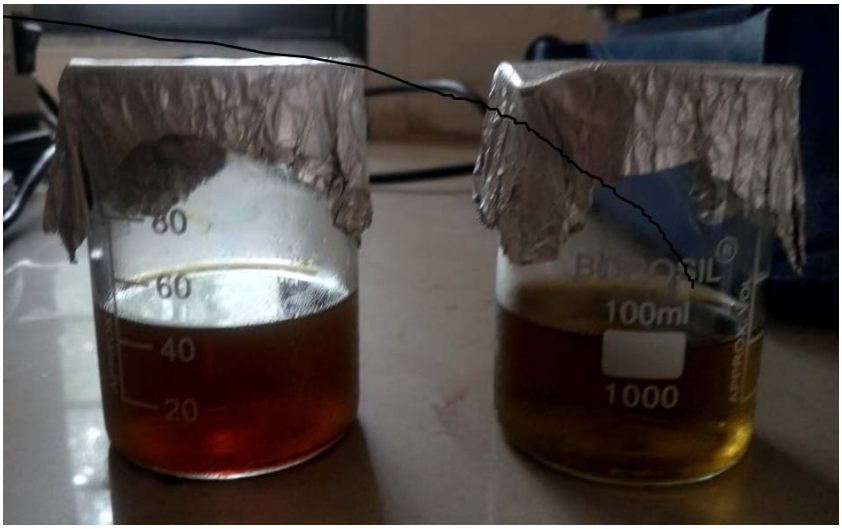
c) Preparation of Active Edible Films

2%v/v aqueous calcium chloride solution at a concentration of 15 ml per 100ml of solution was added to all film forming solutions separately with continuous stirring to improve the physical properties of films. The solutions were casted onto petri plates and were allowed to dry to form six different types of films viz T_1, T_2, T_3, T_4, T_5, T_6 from S_1, S_2, S_3, S_4, S_5, S_6 respectively. The dried films were then removed carefully from the petri plates and stored in desiccators until being used for further studies.

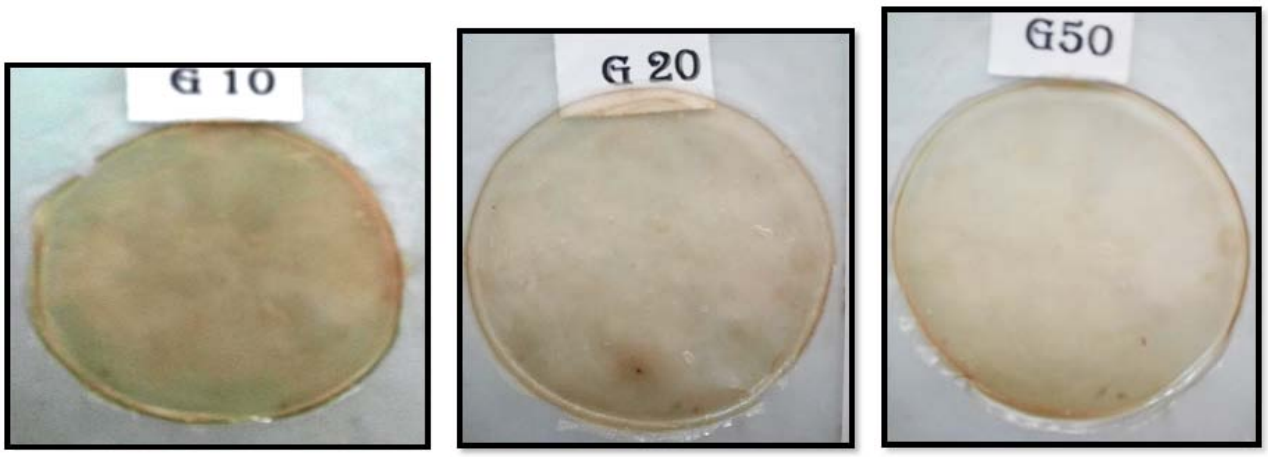
The films were evaluated for different parameters and the results were analysed through SPSS (20.0) with n = 6.



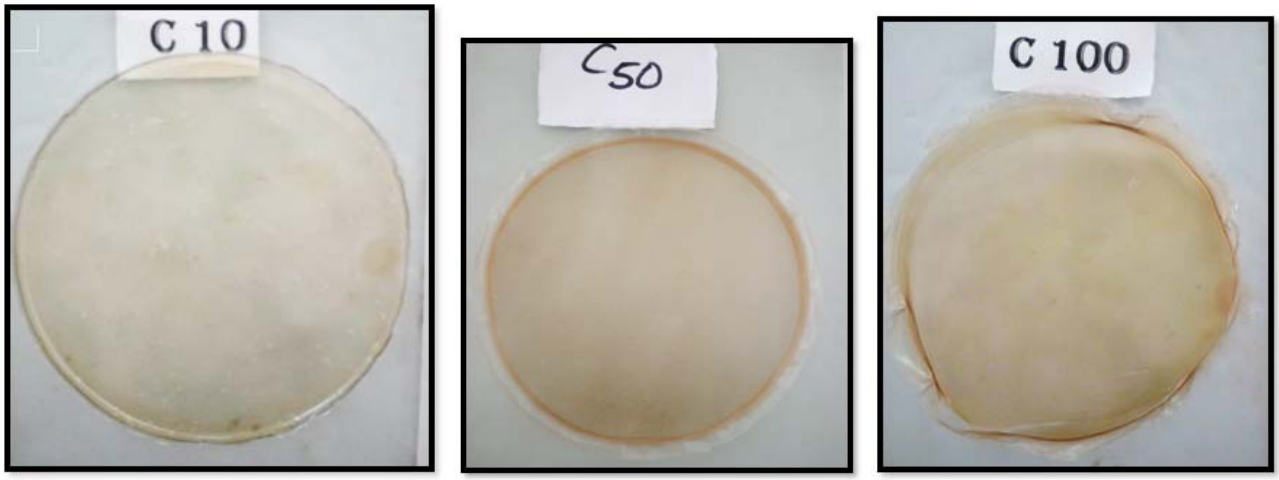
Preparation of nanoemulsion by ultrasonication



Nanoemulsions of Ginger essential oil (left) and Cardamom essential oil (right)



Sodium alginate films incorporated with different concentrations of ginger essential oil nanoemulsions



Sodium alginate films incorporated with different concentrations of cardamom essential oil nanoemulsions

The thickness of each film was measured in *microns* by electronic micrometer (0-25 mm) with an accuracy of 0.001 mm..The average thickness for at least ten random locations has represented as film thickness. The grammature was estimated as per the procedure demonstrated by Geraldine *et.al* (2008).

The water vapour permeability (WVP) of the films was measured gravimetrically based on ASTM E96-92 method as described by Casariego *et al.* (2009). The film was sealed on the top of a glass permeation cup containing distilled water of 20 ºC ,100% RH; 2.337 & 103 Pa vapour pressure and placed in a desiccator which was maintained at 20° C and 1.5% RH and a vapour pressure of 28.044 Pa containing silica gel. The cups were weighed every hour for a period of 8 h. The water transferred through the film and adsorbed by the desiccant was determined from weight loss of the permeation cups. The slope of weight loss versus time was obtained by linear regression curve.

WVP of the films was calculated as follows:

$$\boldsymbol{water\ vapour\ permeability(WAP)} = \frac{\Delta \boldsymbol{g}}{\Delta \boldsymbol{t}} \times \frac{\boldsymbol{X}}{\boldsymbol{A}.\Delta \boldsymbol{P}}$$

Where,

$\frac{\Delta g}{\Delta t}$ - Rate of weight change (g/h),

x - Film thickness (m),

A - Permeation area (m),

ΔP - Partial pressure difference across the film (4244.9 Pa at 30◦C).

The water sorption of edible sodium alginate films was evaluated by following the method of Lavorgna *et al*. (2010). The film samples were cut into small pieces of 2 cm × 2 cm size and placed in desiccator overnight and weighed to obtain their dry mass. Weighed samples were placed in closed beakers containing 30 ml of water (pH 7) and stored at 25 °C. The swelling evaluated by periodically measuring the weight increment of the samples. The film's wet surface was gently blotted with a tissue paper before weighing each time. The water gain of each sample was calculated by using following formula:

$$\boldsymbol{Water\ gain}(\%) = \frac{\boldsymbol{weight\ of\ wet\ film - weight\ of\ dry\ film}}{\boldsymbol{weight\ of\ dry\ film}} \times \boldsymbol{100}$$

Transmission and opacity of the films were evaluated according to the method of Tunc and Duman (2010). The films were cut into rectangular pieces and were placed in the spectrophotometer cell. An empty compartment was used as a reference in the measurements. The light barrier properties of the film samples were measured by scanning the samples at wavelengths between 200 and 800 nm using a UV spectrophotometer. Procedure was repeated for three replicates of each film. The opacity was calculated using the following equation:

$$\boldsymbol{Opacity} = \frac{\boldsymbol{ABS600}}{\boldsymbol{X}}$$

Where,

ABS600 - value of absorbance at 600 nm,

X - film thickness in mm.

In vitro assessment of the antibacterial activity of essential oil nano emulsions was carried out in accordance with the method of Wang et al 2015 with slight modification. In brief, 6 hrs cultures of S.aureus and E.coli were diluted McFarland standard 1 i.e., 0.5 to adjust a microbial count of approximately 1×10^8 cfu/mL. 0.1% Peptone water was sterilized and serial dilutions were prepared and kept for overnight for sterility checking.1 ml of test microbial solution that is adjusted to McFarland standard 1 was taken in to test tube

containing 9ml peptone water and serial dilutions were made. For testing of antibacterial activity the nano emulsions of ginger and cardamom essential oils were aseptically prepared and the 10, 20, 50 µl of giner essential oil nanoemulsion and 10, 50, 100 µl of cardamom essential nanaemulsion were transferred to test tubes containing serially diluted test microbe solution and incubated for 24 h at 37 ^{0}C. Distilled water was used as control. For determination of the colonies, the incubated samples with different serial dilutions were plated onto agar plates (plate count agar) and incubated for 24 h at 37 ^{0}C. Subsequently, colonies were counted for E.coli and S. aureus for each concentration of essential oils and total cfu (colony forming units) was determined, and growth reduction calculated according to Eq. (1). A logarithmic microbial growth reduction of less than 0.5 represents no antibacterial activity. Values between 0.5 and 1 are rated as a slight, values greater than 1 and less or equal to 3 as a significant, and a log reduction greater than 3 as a strong antibacterial activity.

Log growth reduction $_{(24h)}$ = log cfu (control) $_{(24h)}$ - log cfu (sample) $_{(24h)}$ ..... (1)

The anti-microbial activity was determined for the films (T_1 to T_6) by the agar diffusion method, of Pelissari *et al.* (2009).The edible films were aseptically cut in to 2- mm discs and placed on muller hinton agar plates spreaded with 0.1 ml of inocula with $10^5 - 10^6$ CFU/ml of bacterial culture, standardised against McFarland scale. The plates were incubated at 37±1°C for 24 h. The diameter of the zone of inhibition around the discs was measured and equated against an ABST zone of inhibition scale and compared with standard antibiotic zones.

The antioxidant activity was determined by DMPD free radical scavenging assay as described by Fogliano *et al*. 1999. The compound N, N-dimethyl-1, 4-diaminobenzene (DMPD) is converted in solution to a relatively stable and coloured radical form by the action of ferric salt. After addition of a sample containing free radicles, these are scavenged and as a result of this scavenging, the coloured solution is decolourized.

d) Preparation of DMPD solution

DMPD, 100 mM, was prepared by dissolving 209 mg of DMPD in 10 ml of deionised water; 1 ml of this solution was added to 100 ml of 0.1 M acetate buffer, pH 5.25, and the coloured radical cation ($DMPD^+$) was obtained by adding 0.2 ml of a solution of 0.05 M ferric chloride (final concentration 0.1 mM). One millilitre of this solution was directly placed in a 1-mL plastic cuvette and its absorbance at 505 nm was measured. An optical density of 0.900 (0.100 unit of absorbance was obtained and it represents the uninhibited signal. The optical density of this solution, which is freshly prepared daily, is constant up to 12 h at room temperature.

e) DMPD Reagent preparation

Solution 1: acetate buffer (0.2 mol·L-1, pH 5.25)

1a) 2.17 g of sodium acetate trihydrate was dissolved in 80 ml of ACS water.

1b) 300 µl of concentrated acetic acid (>99.5% v/v) was diluted to a volume of 20 ml with ACS water.

These two solutions were mixed to reach the pH 5.5

Solution 2: 0.74 mmol·L-1 ferric chloride: 1 mg of $FeCl_3.6H_2O$ was dissolved with ACS water to a volume of 5 ml.

Solution 3: (36.7 mmol·L-1 DMPD) 25 mg of DMPD was dissolved in 5 ml of ACS water. This solution must be prepared at the time of use due to its low stability.

These three solutions (solutions No. 1, 2 and 3) were mixed in a 20:1:1 (v/v/v) ratio.

A 2.95 ml volume of above reagent was pipetted into a plastic cuvette. Then 50µl of sample was added and absorbance was measured at 505 nm wavelength after 10 minutes at 25 °C. Standard was prepared by adding 2.95 ml of DMPD reagent and 50 µl of trolox solution and Antioxidant activity was expressed as µg/ml of trolox equivalent.

$$\boldsymbol{DMPD\ scavenging\ effect(D.SE\%) = \left(1 - \frac{A_s}{A_c}\right) \times 100}$$

Where,

A_s – absorbance of sample,

A_c – absorbance of the standard.

$$\boldsymbol{Trolox\ equivalent(\mu g/ml) = \frac{D.SE\ \%}{0.7293} - 1.3857}$$

f) Tensile Strength and % Elongation at Break

The tensile strength and % elongation at break was measured following the procedure demonstrated by Soni A *et.al* (2016). The mechanical properties of the films were measured by means of its tensile strength (TS) and % elongation at break (EAB).

The TS value of the edible film was recorded as per the method of Berry and Stiffler 1981.The TS of the film was measured with texturometer. Each film was cut into 8 × 2 cm strips and then mounted between grips onto the texturometer and stretched until they broke. Six observations were recorded for each sample to obtain the average value of shear force in $\boldsymbol{newton/cm^2 (N/cm^2)}$

Elongation at break was measured according to the method of Soni A *et.al* (2016). for calculation of per cent elongation at break, films were cut into 8 × 2 cm strips, and fixed on a manually formed scale. One end of the film was fixed and other end is stretched manually until it was broken. The EAB of the films was calculated as follows:

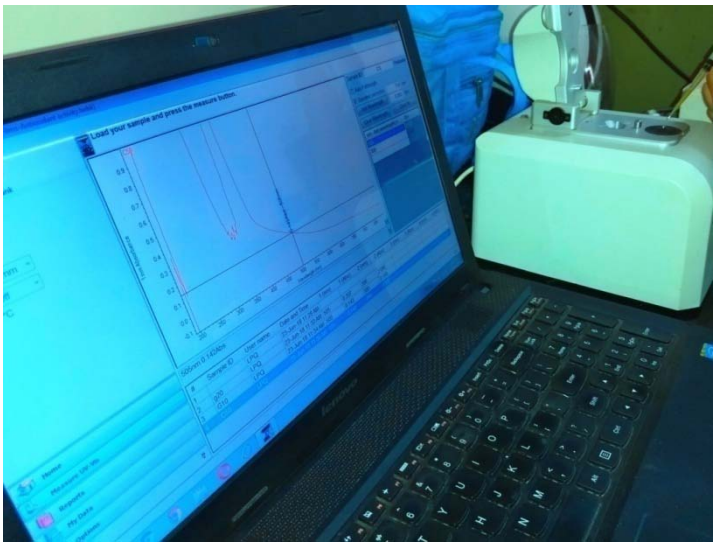
$$\boldsymbol{elongation\ at\ break(\%) = \frac{B - A}{A} \times 100}$$

where,

A – intial length of the film and

B – Final length of the film after stretching/at the time of break.

The results of the above parameters were analysed and depending on the activity of the films, the best film was selected and the selected film was proved for its efficacy extend in the shelf life of the product packed in it. The data was subjected to statistical analysis using SPSS IBM, version 20.0.(n=6)



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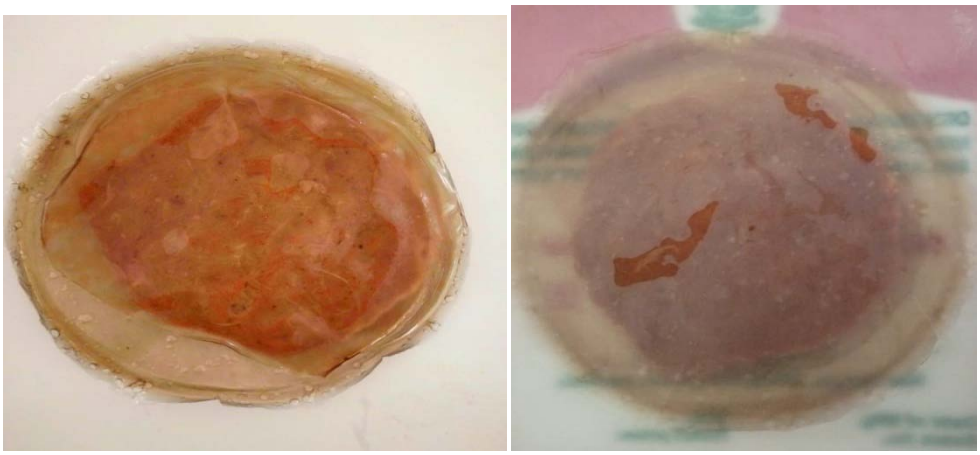

Product Stored in the film

III. Results & Discussion

Thickness of edible film is related to transperancy, water vapour permeability and mechanical properties of the films. (Ejaz et al.2018). The mean thickness and grammature values of both GEON and CEON incorporated films were significantly ($P<0.05$) higher than the control films. The films incorporated with CEON had significantly higher thickness and grammature values than GEON incorporated films. Irrespective of the type of essential oil incorporated the mean thickness and grammature of the films were increased with increasing concentrations of active ingredients incorporated. This might be due to the formation of thin layer over the surface of film due to the hydrophobic interactions between the oils and polysaccharide film (Ejaz et al.2018). The results obtained in the present study were in accordance with Ejaz et al. (2018) in bio nanocomposite gelatin film with clove oil and zinc oxide nano rods and Benavides et al. (2012) in alginate films with oregano oil.

Water vapour permeability measures the diffusion of water molecules through the cross section of the film and can give an estimation of its barrier property. To prevent dehydration of foods, films used as packaging or coating must control the moisture transport from the product to the environment. Hence WVP of edible films should as low as possible (Fabra et al.2008). Generally WVP depends on the diffusivity and solubility of water molecules in the film matrix (Wang et al. 2013).

The mean water vapour permeability of films incorporated with 100 μl of ginger emulsion oil nano emulsion was significantly ($P<0.05$) higher than the other films. The mean WVP of films incorporated with CEON was significantly ($P<0.05$) lower than GEON incorporated films and control. In GEON films, the increase in the concentration of nano emulsion water vapour permeability values were increased significantly ($P<0.05$). This might be due to an increase in the number and size of the holes appearing in the polymer matrix (Chen et al.2016). The increase in the concentration of CEON incorporation leads to a decrease in the water vapour permeability of the films. This might be due to the hydrophobic nature of essential oil that inhibits the water transmission across the film. The WVP of polymer films depends on the hydrophilic and hydrophobic ratio of the film constituents. This could be the possible reason for variation in WVP trend in different treatments (Soni et al. 2016). These results were well in accordance with those of Soni et al. 2016 in oregano and thyme oil incorporated carrageenan based edible films for packaging of chicken patties and Chen et al. 2016 with chitosan films containing Cinnamaldehyde nanoemulsions.

The films water solubility can influence its use for protection of the packaged product from the external environment. The film might be water insoluble especially when applied to high moisture foods like meat (Giteru et al. 2017).

The mean water sorption of both GEON and CEON incorporated films was significantly ($P<0.05$) lower than the control films. The films incorporated with CEON had significantly ($P<0.05$) lower water sorption values than GEON incorporated films. The reduced film solubility in essential oil nano emulsions incorporated films might be due to its hydrophobicity (Soni et al. 2016). Irrespective of the type of essential oil incorporated the mean water sorption kinetic values of the films was decreased with increasing concentration of active ingredients incorporated. This might be due to the higher degree of substitution of water by oil droplets which could lower hydrophilicity of film, thereby lowering the soluble matter of films (Dammak et al. 2017).The results were well in accordance with those of Soni et al. (2016) in essential oil incorporated carrageenan based edible films and Rostamzad et al. (2016) in fish protein films with nanoclay and transglutaminase for food packaging.

Films with lower light transmission and higher opacity values could prevent oxidation induced by UV light in a food system (Rostamzad et al 2016). The mean opacity values of both GEON and CEON incorporated films was significantly ($P<0.05$) higher than the control films. The films incorporated with CEON had significantly ($P<0.05$) higher opacity than GEON incorporated films. Irrespective of the type of essential oil incorporated the mean opacity of the films was increased with increasing concentrations of active ingredients incorporated. This could be attributed to decrease in light transmission and due to the light scattering or interference of light passage by the lipid droplets in essential oils (Ejaz et al. 2018).

This would indicate that alginate films containing essential oils could act as good barriers for UV and visible light thereby reducing the light induced lipid oxidation. These results were in accordance with those of Ejaz et al. (2018) in gelatin composite films with clove essential oil and zinc oxide nanorods and Soni et al. (2016) in essential oil incorporated carrageenan based edible films.

Anti oxidant activity evaluates the capacity of the films to scavenge free radicals. Determination of radical scavenging activity of films is important because of harmful effects of free radicals in foods and biological systems (Noori et al.2018).

The mean anti oxidant activity in μg/ml trolox equivalent of active edible films incorporated with different concentrations of GEON and CEON was significantly ($P<0.05$) higher than the control films. The films incorporated with CEON had significantly ($P<0.05$) higher anti oxidant values than GEON incorporated films. This could be due to the presence of chemical components such as α-zingiberene and α-terpinyl acetate in ginger and cardamom essential oils which were known to have strong anti oxidant activity (Noori et al. 2018, Kandikattu et al.2017). Irrespective of the type of essential oil incorporated the mean anti oxidant activity in μg/ml of trolox equivalent was increased with increasing concentrations of active ingredients incorporated. The formation of nano emulsion had decreased the degradation of essential components coupled with increasing the surface area of essential oils in the film thereby achieving a fast and efficient free radical absorption with increasing concentrations of essential oil incorporation (Noori et al. 2018).

The higher antioxidant of CEON incorporated films might be due to the assembly of large amounts of essential oil on to the alginate film due to the strong hydrophobic interactions between polymer and essential oil (Wang et al. 2017). The results were well in agreement with Noori et al (2018) in chicken breast fillets added with nanoemulsion of Ginger Essential oil and Kandikattu et al. (2017) on anti inflammatory and anti oxidant effects of Cardamom.

Anti microbial activity was evaluvated against gram- negative *(E.coli*) and gram- positive bacteria (*S.aureus*) to gain a better understanding of the mechanisms of anti bacterial activity and were expressed in log CFU/ml. The mean *E.coli* and *S.aureus* counts of both GEON and CEON incorporated films were significantly ($P<0.05$) lower than the control films. The films incorporated with CEON had significantly ($P<0.05$) lower counts than GEON incorporated films. Irrespective of the type of essential oil incorporated the

mean *E.coli* and *S.aureus* counts were decreased with increasing concentration of active ingredients incorporated, into the films and especially with the formulations T_3 and T_6 . The mean log reduction of GEON and CEON incorporated films against both *E.coli* and *S.aureus* were 1.66, 2.87 and 1.54, 2.8 respectively. The log reduction was significant for both *E.coli* and *S.aureus* but high with *S.aureus*. The mean log reduction in the bacterial counts might be due to the presence of α – zingiberene, β- sesquiphellandrene and zingiberenol in ginger essential oil (Noori et al. 2018) and α-terpinyl acetate and 1-8 cineole in cardamom essential oil (Mejdi et al 2016). The mode of action of the Essential oils against bacteria is thought to be due to the hydrophobic nature of oils, the active components cross bacterial membrane easily leading to loss of ions which results in reduction of the electric potential of membrane and loss of function of protons, thus decreasing ATP which promotes cell death of bacteria. A reduction in droplet size of essential oils by nano emulsion formation would allow anti microbial compounds to penetrate faster in to the bacterial cells, thus a higher anti microbial behavior was observed with increasing concentration of essential oil nano emulsion incorporation. The results were in accordance with Noori et al. (2018) with nano emulsions of Ginger essential oil on chicken breast fillets, Kandikattu et al.(2017) with Cardamom essential oil and Trujillo et al.(2015) with lemon grass essential oil.

Edible packaging films must with stand the normal stresses encountered during its application and the subsequent shipping, storage and handling of the food. To maintain its integrity and barrier properties, high puncture strength is required. Therefore film tensile strength is of most important in accordance with the intended application of the film. The mean Tensile strength and percent elongation at break values of both GEON and CEON incorporated films were significantly ($P<0.05$) higher than the control films. The films incorporated with CEON had significantly ($P<0.05$) higher Tensile strength and percent elongation at break than GEON incorporated films. Irrespective of the type of essential oil incorporated the mean tensile strength and per cent elongation at break of the films increased with increasing concentration of active ingredients incorporated. This might be due to a kind of cross linking between chemical components of essential oils and alginate base matrix which developed a resistant and elastic film matrix structure (Dammak et al. 2017). The Nano emulsions with droplets of oil loaded into sodium alginate were liquid at 25^0 C and its presence in the film structure at nanoscale size can easily be deformed enhancing the film flexibility, thereby increasing the tensile strength and percent elongation of the films with increasing concentration of essential oil nano emulsion incorporation (Dammak et al. 2017). The results were in accordance with Dammak et al. 2017 in properties of active gelatin films with rutin loaded nano emulsions.

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Acknowledgments

Contributors to the research other than authors credited should be mentioned in Acknowledgments. The source of funding for the research can be included. Suppliers of resources may be mentioned along with their addresses.

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The following is the official style and template developed for publication of a research paper. Authors are not required to follow this style during the submission of the paper. It is just for reference purposes.

Manuscript Style Instruction (Optional)

- Microsoft Word Document Setting Instructions.
- Font type of all text should be Swis721 Lt BT.
- Page size: 8.27" x 11'", left margin: 0.65, right margin: 0.65, bottom margin: 0.75.
- Paper title should be in one column of font size 24.
- Author name in font size of 11 in one column.
- Abstract: font size 9 with the word "Abstract" in bold italics.
- Main text: font size 10 with two justified columns.
- Two columns with equal column width of 3.38 and spacing of 0.2.
- First character must be three lines drop-capped.
- The paragraph before spacing of 1 pt and after of 0 pt.
- Line spacing of 1 pt.
- Large images must be in one column.
- The names of first main headings (Heading 1) must be in Roman font, capital letters, and font size of 10.
- The names of second main headings (Heading 2) must not include numbers and must be in italics with a font size of 10.

Structure and Format of Manuscript

The recommended size of an original research paper is under 15,000 words and review papers under 7,000 words. Research articles should be less than 10,000 words. Research papers are usually longer than review papers. Review papers are reports of significant research (typically less than 7,000 words, including tables, figures, and references)

A research paper must include:

a) A title which should be relevant to the theme of the paper.
b) A summary, known as an abstract (less than 150 words), containing the major results and conclusions.
c) Up to 10 keywords that precisely identify the paper's subject, purpose, and focus.
d) An introduction, giving fundamental background objectives.
e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition, sources of information must be given, and numerical methods must be specified by reference.
f) Results which should be presented concisely by well-designed tables and figures.
g) Suitable statistical data should also be given.
h) All data must have been gathered with attention to numerical detail in the planning stage.

Design has been recognized to be essential to experiments for a considerable time, and the editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned unrefereed.

i) Discussion should cover implications and consequences and not just recapitulate the results; conclusions should also be summarized.
j) There should be brief acknowledgments.
k) There ought to be references in the conventional format. Global Journals recommends APA format.

Authors should carefully consider the preparation of papers to ensure that they communicate effectively. Papers are much more likely to be accepted if they are carefully designed and laid out, contain few or no errors, are summarizing, and follow instructions. They will also be published with much fewer delays than those that require much technical and editorial correction.

The Editorial Board reserves the right to make literary corrections and suggestions to improve brevity.

Format Structure

It is necessary that authors take care in submitting a manuscript that is written in simple language and adheres to published guidelines.

All manuscripts submitted to Global Journals should include:

Title

The title page must carry an informative title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) where the work was carried out.

Author details

The full postal address of any related author(s) must be specified.

Abstract

The abstract is the foundation of the research paper. It should be clear and concise and must contain the objective of the paper and inferences drawn. It is advised to not include big mathematical equations or complicated jargon.

Many researchers searching for information online will use search engines such as Google, Yahoo or others. By optimizing your paper for search engines, you will amplify the chance of someone finding it. In turn, this will make it more likely to be viewed and cited in further works. Global Journals has compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

Keywords

A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, “What words would a source have to include to be truly valuable in a research paper?” Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

Numerical Methods

Numerical methods used should be transparent and, where appropriate, supported by references.

Abbreviations

Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

Formulas and equations

Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

Tables, Figures, and Figure Legends

Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.

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Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

Preparation of Eletronic Figures for Publication

Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/ photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution at final image size ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs): >350 dpi; figures containing both halftone and line images: >650 dpi.

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Tips for writing a good quality Medical Research Paper

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.

6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.

Mistakes to avoid:

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- Separating a table, chart, or figure—confine each to a single page.
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- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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