Effects of *Trichilia monadelpha* (Thonn.) J. J. De Wilde (MELIACEAE) Bark Extracts on Ulcerative Colitis, Colonic Microflora and Wound Healing in Wistar Rats

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT FOR A DEGREE IN M. PHIL PHARMACOLOGY

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BY

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DEDICATION

This work is whole heartedly dedicated to God the maker of all good things, to my family, friends and well-wishers.

DECLARATION

I, Mr. Patrick George Ayande, do hereby declare that this dissertation is the result of my original research carried out in fulfilment for the award of my M. Phil Degree in Pharmacology at the Kwame Nkrumah University of Science and Technology. I also affirm that, no part or whole of it has gone in for any such award in any educational setting, and that I am solely responsible for any commission or omission.

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ABSTRACT

Trichilia monadelpha (Thonn.) de Wilde, has been reported to have therapeutic effect against microbes, dysentery, dyspepsia, sores and ulcers. However, no scientific basis is established of such claims. Thus, this study sought to determine the effectiveness of *Trichilia monadelpha* bark extracts on surface wounds and ulcerative colitis as well as the extent to which it could displace colonic microflora. Generally, Wistar rats of comparable age and weight were housed 5 per cage in 6 groups for each experimental set-up. The excision wound healing and indomethacin/acetic-acid induced animal models of ulcerative colitis were employed in this study. All extracts for the treatment of ulcerative colitis were administered orally, while wound treatment was carried out with topical formulations. In the indomethacin-induced ulcerative colitis, colons of animals treated with the ethanolic extract revealed persistent mucosal ulceration in the disease control and 30 mg/kg but not the 100 mg/kg and 300 mg/kg dose groups. For the acetic acid-induced ulcerative colitis, the aqueous extract significantly restored mucosal integrity in the 100 mg/kg and 300 mg/kg dose treated groups relative to the ulcerated and oedematous mucosae of 30 mg/kg treated group, pointing to a marked response to treatment in a dose dependent fashion. The petroleum ether extract manifested crypt abscesses and globular mucosae in the 100 mg/kg and 300 mg/kg dose treated groups of acetic acid-induced colitic rats, while the ethyl acetate extract showed serrated, crypt and oedematous mucosae among the 100 mg/kg and 300 mg/kg dose groups, evident of persistent disease. The role of *Trichilia* on colonic microflora in colitic rats suggested a dose dependent activity. There was a 24%, 12% and 8% microbial infestation among animals of the 30 mg/kg, 100 mg/kg and 300 mg/kg treated groups relative to a 32% and 24% occurrence in disease and normal controls respectively. Therefore, *Trichilia* was found to be detrimental to colonic microflora.

In the case of excision wounds, the aqueous extract of *Trichilia* showed appreciable rate of healing in the 3% and 10% treated groups but not in the 30% dose treated group. On the other hand, rate of wound healing was dose dependent and comparable to the standard treatment among the ethanolic extract treated groups. Most wounds had completely healed by day 13. But the petroleum ether extract showed a relatively poor rate of wound healing with sepsis resulting into chronic wounds. The rate of wound healing in the ethyl acetate extract group was extremely poor, registering 100% mortality in the 30% treated group, characteristic of ineffective treatment. An anti-oxidant assay established the presence of anti-oxidant properties in the aqueous, ethanolic, and ethyl acetate extracts but not the petroleum ether extract. In conclusion, the aqueous extract of *Trichilia* proved to have a strong anti-oxidant property and was highly effective in the treatment of ulcerative colitis but slightly effective in excision wounds, while the ethanolic extract had appreciable anti-oxidant property and was effective in the treatment of ulcerative colitis but highly effective in the healing of excision wounds. The petroleum ether and ethyl acetate extracts did not prove to be effective treatment options in both cases. *Trichilia* was also found to displace colonic microfloral balance and could be effective against infectious colitis.

ACKNOWLEDGEMENT

I very much acknowledge the supervisory role of Professor Eric Woode, the laboratory technicians of Pharmacology, KNUST, and Mr. Peter K. Obyn of Biological sciences, UCC, as well as the many others whose support cannot be mentioned.

To this, I pray yee all, the favour of God, through Christ our lord who lives and reigns with the father and the spirit one God forever and ever.

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ABBREVIATIONS

5-ASA	5-Amino-Salicylic Acid
AICD	Activation-Induced Cell Death
AnxA1	Annexin A1
ARE	Adenine/uracil Rich element
BBA	Brucella blood agar
bFGF	Basic Fibroblast Growth Factor
BHI	Brain heart infusion
cAMP	Cyclic Adenosine Mono Phosphate
CFU	Colony Forming Unit
CoA	Co-enzyme A
COX	Cyclooxygenase
d	day
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DSS	Dextran Sulphate Sodium
EGF	Epithelial Growth Factor
EMB - agar	Erosin-methylene blue
ExW	Excision Wound
GAFCO	Ghana Agro Food company
GAG	Glycosaminoglycan
GCs	Glucocorticoids
GI	Gastroenterology Intestinal
GIT	Gastroenterology Intestinal Tract
GM–CSF	Granulocyte Macrophage–Colony-Stimulating Factor
GSH	Glutathione
HDAC	Histone Deacetylase
HOCl	Hypochlorous Acid
H_2O_2	Hydrogen Peroxide
Hsp	Heat Shock Protein

IBD	Inflammatory Bowel Disease control
(ICAM) 1	Intercellular Adhesion Molecule 1
IFN Interferon	IFN Interferon
IL Interleukin	IL Interleukin
ITFs	Intestinal Trefoil Factors
KNUST	Kwame Nkrumah University of Science and Technology
KO	Knockout
LAK	Lymphokine Activated Killer
LT	Leukotrienes
LTB4	Leukotriene B4
MAPK	Mitogen Activated Protein Kinase
MCP	Monocyte Chemoatractant and Protein 1
MDA	Malondialdehyde
MHC	Major Histocompatibility Complex
MMPs	Matrix Metallo Proteinases
MPO	Myeloperoxidase
NAC	N-acetyl-cysteine
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NF - kB	Nuclear Factor – kappaB
NK	Natural Killer
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
O^{2-}	Superoxide
^{-}OH	Hydroxyl Radical
OHP	Hydroxyproline
$ONOO^{-}$	Peroxynitrite
PGs	Prostaglandins
PD E	Prostaglandin E
PFGE	Pulse Field Gel Electrophoresis
Pg–Ps	Peptidoglycan–Polysaccharide
PMN Cells	Polymorphic Nuclear Cells

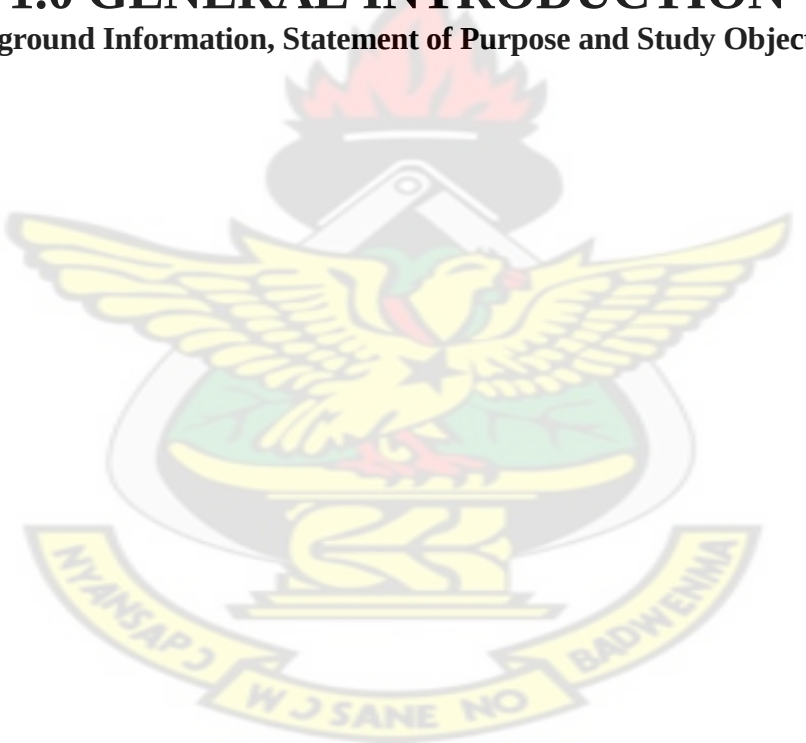
PPI	Proton Pump Inhibitor
PVA	Polyvinyl Alcohol
RFLP	Restriction fragment length polymorphism
RGCA	Rumen fluid-glucose-cellobiose agar
ROM	Reactive Oxygen Metabolites
ROS	Reactive Oxygen Species
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
SCID	Severe Combined Immunodeficiency
SH	Sulfhydryl
STAT	Signal Transduction and Activator of Transcription
Rwh	Rate of wound healing
TAP - PCR	Triplet Arbitrary Primed PCR
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid reactive spieces
TCA	Trichloro-Acetic Acid
TCR	T cell Receptor
TLC	Thin Layer Chromatographic
TLR	Toll-Like Receptors
Th	T helper
TMB	*Trichilia* Monadelpha Bark
$TMBE_{Et}$	*Trichilia* Monadelpha Bark Extracted in Ethanol
$TMBE_{Aq}$	*Trichilia* Monadelpha Bark Extracted in Aqueous
$TMBE_{Pt}$	*Trichilia* Monadelpha Bark Extracted in Petroleum Ether
$TMBE_{Ea}$	*Trichilia* Monadelpha Bark Extracted in Ethyl Acetate
TNF	Tumour Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TSI	Triple sugar iron
UC	Ulcerative Colitis
UCC	University of Cape Coast

UTR	Untranslated Region
(VCAM)-1	Vascular Cell Adhesion Molecule
Wc	Wound Contraction
Ws	Wound size
ΔWd	Change in wound diameter

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Background Information, Statement of Purpose and Study Objectives



1.1 Background Information

Ulcerative wounds and surface wounds are epithelial defects, resulting from mechanical and thermal damage or are otherwise traceable to the presence of an underlying pathophysiological condition. Wounds are generally classified according to degree of insult into superficial wounds; injury to the epidermis, and deep-seated wounds; injury to the epidermal, dermal, and subcutaneous layer. A wound is described as acute if it heals under 8 weeks, and chronic when it lasts much longer. Acute colitis is considered to be induced by innate immunity, but not acquired immunity, because it also occurs in severe combined immunodeficiency (SCID) subjects. However, chronic colitis is considered to be caused by lymphocytes that are activated by the cytokines secreted from the activated macrophages (Hibi *et al.*, 2002).

Characterised by the development of intestinal inflammation, resulting most probably from interaction of the immune system, genetic susceptibility, feeding habits and drug interactions, ulcerative colitis as a worldwide, chronic inflammatory bowel disease (IBD) affects the rectal and colonic mucosa. It may occur in people of any age, but most often it starts between ages 15 to 30 and affects men and women equally but appears to run in some families (Tai *et al.*, 2007).

Wound healing on the other hand, is a dynamic process in which central tissue movements associated with repair, such as angiogenesis, granular tissue formation, and re-epithelialisation act together to replace necrotized and/or damaged tissue and to re-establish its integrity. Therefore, irrespective of the aetiological agent, wound healing proceeds in four stages; inflammation, migration, proliferation and remodelling (Frank and Kampfer, 2003).

1.2 THE PLANT

1.2.1 Plant Identification

Name	*Trichilia monadelpha* (Thonn.) J. J. De Wilde
Traditional Names	Otanduro in Twi, Tenuba in Nzema
Family	MELIACEAE
File #	FPP/079/10
Locality	Tepa, Brong-Ahafo, Ghana
Collector	P. G. Ayande
Collection Date	20/08/2010
Verified By	Dr. Kofi Annan
Repository	Herbarium of Faculty of pharmacy & Pharmaceutical Sciences, KNUST

1.2.2 Plant Ecology and Ethnopharmacology

Trichilia monadelpha of the family MELIACEAE, is a 20-60 ft. high typical woody plant, flora of West Tropical Africa and is common in moist under-storey of rain forest, especially in secondary re-growth types. *Trichilia* bark extracts has been reported to have therapeutic effect against microbes, dysentery, dyspepsia, sores/ulcers (Busia, 2007).

1.2.3 Plant Description

It has a grey bark, slash dark pink with slight exudates of whitish latex. Its flowers are greenish yellow, with 2 cm globose fruits occurring in clusters and buffed colour, while its 15 cm long pinnate leaves are oblong with numerous lateral nerves (Irvine 1961). A kin observation pointed to the fact that *Trichilia* is a dioecious plant with a characteristic single trunk male plant of about 50 – 60 ft and divergent trunk female counterpart of about 10 – 20 ft with a relatively broad canopy.

Figure 1.1: Phenotype of *Trichilia monadelpha* (Thonn.) J. J. De wilde

1.3 STATEMENT OF PURPOSE AND STUDY OBJECTIVES

1.3.1 Statement of Purpose

Most therapies for ulcerated-wound healing do not only show limited benefits but also manifest severe undesirable side effects (Hendrickson *et al.*, 2002). Moreover, these pharmaceutical products are too expensive for the poorer populations (Borrelli and Izzo, 2000). Consequently, there is a need for safer, effective and affordable alternative therapies with fewer side effects.

Trichilia monadelpha* (Thonn.) de Wilde*; has been reported to have therapeutic effect on arthritis, dysentery and dyspepsia. Its antitussive and wound healing properties as indicated in the Ghana Herbal Pharmacopoeia (Busia, 2007), cannot be over emphasized. Though herbalists of Sub-Saharan Africa employ *Trichilia* decoctions and ethanolic tinctures in the management of diseases including inflamed wounds and ulcers (Busia, 2007), no pharmacological data on *T. Monadelpha* exist. Moreover, its association with ulcerated-wound healing is unknown. Thus this study sought to determine whether ***Trichilia monadelpha*** bark extract will be effective in the initiation and promotion of healing in ulcerative colitis and excision wounds.

Meanwhile, like all other antimicrobial agents *Trichilia* could cause disruption of microfloral balance, leading to decreased colonisation resistance, colonization by opportunistic pathogens and alterations in the metabolic benefits of the microflora. As such, the need to investigate whether *Trichilia* bark extract can vary intestinal normal flora in colitic rats also became necessary. Especially that *Trichilia* extracts are administered orally for the treatment of colitis.

1.3.2 Objectives of the Study

1.3.2.1 General Objective

Generally, the study sought to determine the effect of *Trichilia monadelpha* bark extracts on colonic ulcers, their associated disruption of colonic microflora and on excision wounds.

1.3.2.2 Specific Objectives

Complementary to the above, the study will specifically look to:

1. Ascertain the pathology of ulcerative colitis and excision wounds in rat models
2. Determine the effect of *T. monadelpha* bark extracts on colonic ulcers
3. Determine the effective extract of *T. monadelpha* bark against colonic ulcers
4. Determine the role of the effective extract of *T. monadelpha* bark on colonic microflora
5. Determine the effect of *T. monadelpha* bark extracts on excision wounds
6. Determine the effective formulation of *T. monadelpha* bark on excision wounds

CHAPTER TWO

2.0 PHYTOCHEMISTRY

Plant Collection, Extraction and Phytoscreening

2.1 Plant Collection

The plant parts were sampled from Bomaa, Brong-Ahafo, Ghana ($7^0$05”07.21”N, $2^0$10”10.36”W, elev 233 m) on the 20th day of August, 2010, verified and confirmed by Dr. Kofi Annan, Department of Herbal Medicine, KNUST. A specimen is available in the Faculty of Pharmacy & Pharmaceutical Sciences’ Herbarium, KNUST, voucher number FPP/079/10.

Figure 2.1: *Trichilia monadelpha* bark Sample

2.2 Preparation of Extracts

Freshly harvested *Trichilia monadelpha* bark (TMB) was further chopped into minute chips and sun-dried, then ground into coarse powder. The TMB powder portions were macerated in petroleum ether, ethyl acetate and in 70% ethanol, allowed to percolate and subsequently vaporised to concentrate. Prior to experimentation, the concentrated crude extracts were further dried in hot air oven at 60 ^oC to vaporise the remaining solvents and the resultant referred to as; TMB_{Et} for the ethanolic extract, TMB_{Pt} for the petroleum ether extract, and TMB_{Ea} for the ethyl acetate extract respectively. The aqueous extract on the other hand, powdered TMB was warmed in water at 50 ^oC for 30 minutes, filtered and also vapourised in a hot air-oven at 60 ^oC until a constant weight of TMB_{Aq} (aqueous extract) was obtained.

2.3 Thin Layer Chromatographic (TLC) screening

The presence of saponins, tannins, alkaloids, and glycosides as well as steroids, terpenoids and flavonoids etc, is key in establishing the medicinal properties of *Trichilia* bark. In this study, thin layer chromatographic (TLC) screening was carried out using mixtures of petroleum ether, ethyl acetate and methanol to ascertain the chromogenic reaction of *Trichilia monadelpha* to anisaldehyde. The mobile phase (6:3:1 petroleum ether, ethyl acetate and methanol system) which gave best separation was selected for the chromatographic finger print.

2.4 Anti-Oxidant Assay

A rapid screening antioxidant assay was also carried out for the occurrence of possible components and their anti-oxidant activities. Crude aqueous, ethanolic, petroleum ether and ethyl acetate extracts were reconstituted in methanol and spotted on pre-coated silica gel F_{254} aluminium sheets. The plates were developed in chloroform-methanol (9:1 v/v), sprayed with 20 mg/l solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and the colour change in 30 minutes of spraying constituted positive results (Cuendet *et al.*, 1997).

2.5 RESULTS

2.5.1 Thin Layer Chromatography (TLC)

TLC screening was carried out using mixtures of petroleum ether, ethyl acetate and methanol to ascertain the chromogenic reaction of *Trichilia monadelpha* to anisaldehyde. The mobile phase which gave best separation was (6:3:1 petroleum ether, ethyl acetate and methanol system) selected for the chromatographic finger print as in Fig 2.1.

Figure 2.2: Thin Layer Chromatographic Finger Print; (a) Aqueous Extract (b) Ethanolic Extract (c) Ethyl acetate Extract (d) Petroleum ether Extract. **Note:** Arrow points to direction of constituent migration.

Table 2.1: Phytoscreening

Extract	Phytoscreening			
	No. of Compounds	**No. of Spots**	**Separation**	**Rf Value (mm)**
A. Aqueous	None	0	A1	0.00
			B1	0.40
B. Ethanolic	Two	2	B2	0.91
			C1	0.40
			C2	0.52
C. Ethyl Acetate	Five	5	C3	0.87
			C4	0.94
			C5	1.02
			D1	0.87
D. Petroleum Ether	Three	3	D2	0.94
			D3	1.04

Note: 0 spot = No perceived active compound, 2 spots = Tow perceived active compounds, 3 spots = perceived active compounds, 5 spots = perceived active compounds present in the aqueous, ethanolic, ethyl acetate and petroleum ether extracts respectively.

2.5.2 Rapid Screening Anti-Oxidant Assay

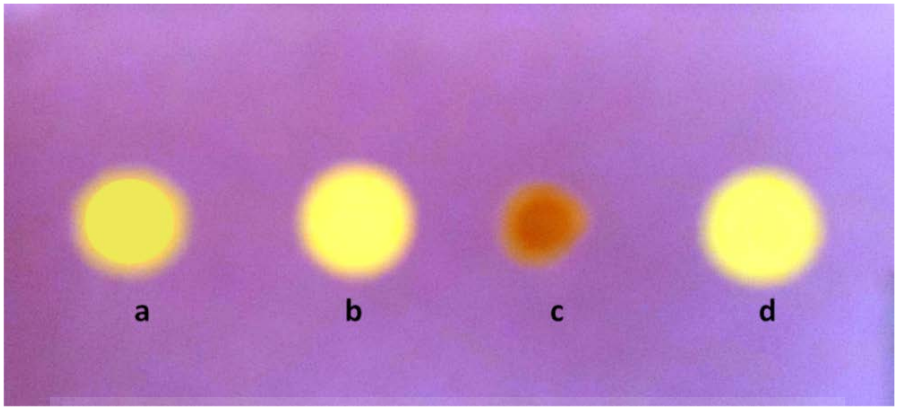


Figure 2.3: Rapid Screening Anti-Oxidant Assay; (a) Aqueous Extract (b) Ethanolic Extract (c) Petroleum ether Extract (d) Ethyl acetate Extract.

Table 2.2: Anti-Oxidant Assay

Extract	**Anti-Oxidant Property**	
	Colour Indicator	**Present/Absent**
a. Aqueous	Yellow	Present
b. Ethanolic	Yellow	Present
c. Petroleum Ether	Brick-Red	Absent
d. Ethyl-Acetate	Yellow	Present

An antioxidant assay employing the rapid screening method of TLC established the presence of anti-oxidant properties in the aqueous, ethanol, and Ethyl acetate extracts but not the petroleum ether extract.

2.6 DISCUSSION

The TLC screening carried out to ascertain the chromogenic reaction of *Trichilia monadelpha* to anisaldehyde. The preferred system, 6:3:1 petroleum ether, ethyl acetate and methanol, for the chromatographic finger print showed no spot for aqueous, three spots for ethanolic, five for ethyl acetate and three spots for the petroleum ether extracts, implying presumably that, there were 5 compounds in the ethyl acetate extract, 3 each in both the ethanolic and petroleum ether extracts and 1 in the aqueous. This separation however, only holds for this solvent system since varying the polarity will vary the outcome.

Generally the anti-oxidant property of *Trichilia* bark extracts as demonstrated in the rapid screening assay was positive for the aqueous, ethanol, and ethyl acetate extracts but not the petroleum ether extract.

As such, it is expected that the $TMBE_{Aq}$, $TMBE_{Et}$ and the $TMBE_{Ea}$ but not the $TMBE_{Pt}$, will most probably ameliorate colonic ulcers by improving colonic oxidative balance resulting from the principal free radicals; superoxide anion (O^{2-}), peroxide (H_2O_2) and the hydroxyl anion (OH^-), thus reducing enzymatic activity of myeloperoxidase and the concomitant effect of a sustained production of reactive oxygen metabolites leading to oxidative injury. The $TMBE_{Aq}$ and $TMBE_{Et}$ with their good anti-oxidant properties would also be effective dressings against acute inflammation in the excision wounds.

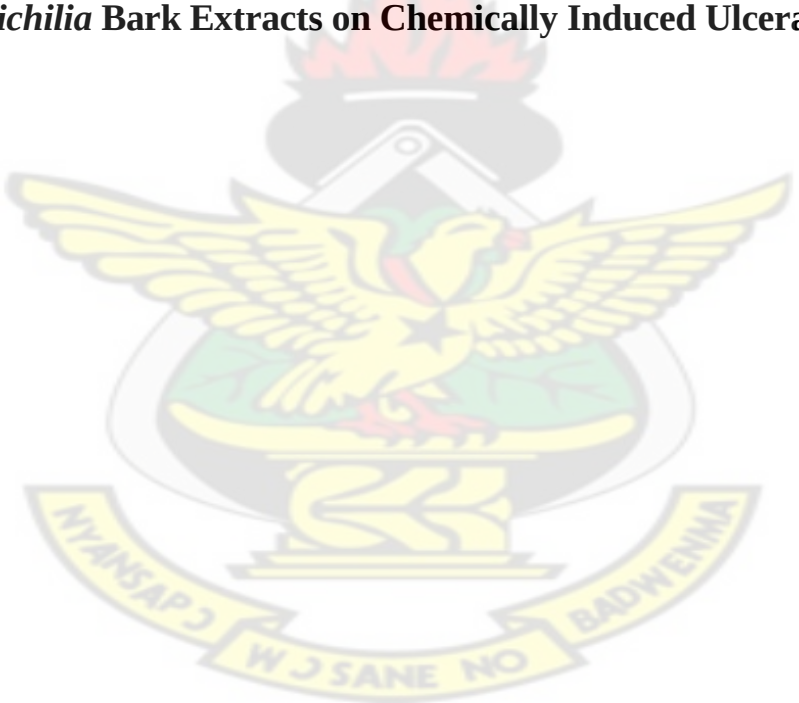
2.7 CONCLUSION

Trichilia monadelpha bark was found to have multiple compounds with varying solubilities and degrees of separations, some of which possessed appreciable anti-oxidant properties. It could therefore be described as being poly pharmaceutical.

CHAPTER THREE

3.0 ULCERATIVE COLITIS

Effect of *Trichilia* Bark Extracts on Chemically Induced Ulcerative Colitis



3.1 INTRODUCTION

3.1.1 Background Information

Ulcerative colitis (UC) is a chronic, idiopathic, inflammatory bowel disease (IBD) of the rectal and colonic mucosa. It is characterised by colonic inflammation, resulting most probably from the infiltration of polymorphonuclear cells, lymphocytes, monocytes, and plasma cells, accompanied by the overproduction of oxygen free radicals, ultimately leading to mucosal alteration and ulceration (Cho *et al.,* 2007). Though the exact pathogenesis is poorly understood, it is established that interaction of the immune system, genetic susceptibility, feeding habits and drug interactions could be responsible (Tai *et al.,* 2007). The most common symptoms are abdominal pain and bloody diarrhoea. But may include; fatigue, weight loss, loss of appetite, body fluids and nutrients. A combination of blood tests, biopsies, radiography & colonoscopy contribute to making a firm diagnosis of UC (Lehne *et al.,* 2004).

The colon, primarily concerned with water resorption, has a quite flat and glandular surface with no villi at all. Its endothelium is covered by a mucus layer, which is associated with the protection of the epithelial surface from mechanical stress and bacterial pathogens as well as luminal components such as stomach acidity and proteolytic enzymes (Faure *et al.,* 2003).

3.1.2 Oxidative Stress and Pathogenesis of Ulcerative Colitis

Colonic injury of all forms may elicit a chronic immune response resulting in cellular proliferation. If the immune response fails to eliminate the inciting agent, proliferation continues in attempt to repair. Continued inappropriate proliferating immune cells produce a variety of cytokines and chemokines that propagate the inflammatory response and enhance the growth and survival of malignant cells (Jackson and Evers, 2009). Dysfunction of such immunologic tolerance is presumed to be a cause of ulcerative colitis (Hibi *et al.,* 2002).

Mitochondria as the power-generating units of the cell and whose primary role is to convert the products of carbohydrate, protein, and fat to carbondioxide ($CO_{2)}$ and water (H_2O), uses key enzymes of the electron transport chain in a process called oxidative phosphorylation, central to the health of a variety of tissues and organs (Johannsen and Ravussin, 2009). Simultaneously, it also generates reactive oxygen species (ROS), such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), the hydroxyl radical (^{-}OH), peroxynitrite ($ONOO^{-}$) and hypochlorous acid (HOCl), which react with biological molecules non-specifically (Rodriguez-Cuenca *et al.*,2010). With time, this excess ROS production may exceed the antioxidant capacity of the mitochondria eventually causing cell damage or death (Johannsen and Ravussin, 2009). Thus, an imbalance between the generation of reactive oxygen species and the endogenous antioxidant defense mechanisms of the colon may be involved in the initiation or aggravation of the inflammatory process. In addition to the oxidative damage by ROS, reactive nitrogen species (RNS) is thought to promote inflammation by mediating chemotaxis of neutrophils and monocytes. Moreover, NO reacts easily with O_2^{-} to generate the $ONOO^{-}$, which is a potent and more toxic oxidant (Horvath *et al.*, 2008).

Inflammed colons would have an altered redox status with decreased glutathione (GSH) levels. Glutathione peroxidases contribute nearly all of the GSH-dependent, H_2O_2-reducing activity in the colonic epithelium, suggesting that these enzymes and sufficient GSH levels are essential for protecting the cells from oxidative stress in the colon (Narushima *et al.*, 2003). Aconitase, found in both the mitochondria and the cytosol, is a target of oxidative stress because of the loss of Fe from the [4Fe-4S] cluster. A decrease in aconitase activity suggests an increase in oxidative stress (Rodriguez-Cuenca *et al.*, 2010). Macromolecular uptake is elevated where mucosal inflammation is present (Faure *et al.*, 2003).

There are a range of endogenous natural antioxidants that are synthesized within the human body, required as non-essential components of the diet, like vitamins E and C, coenzyme Q10 and several polyphenolic compounds (Rodriguez-Cuenca *et al.,* 2010). Most antioxidants are delivered non-specifically to the whole body and to all cell types, thus potentially diluting their efficacy since pathology-associated oxidative stress has a specific cellular location. The production of ROS may not always be an unwanted and damaging process in the body. For example, ROS are used by neutrophils to facilitate the killing of bacteria. ROS also participate in a range of redox signalling pathways, while the levels of certain ROS may act to modulate the activity of signalling pathways (Rodriguez-Cuenca *et al.,* 2010).

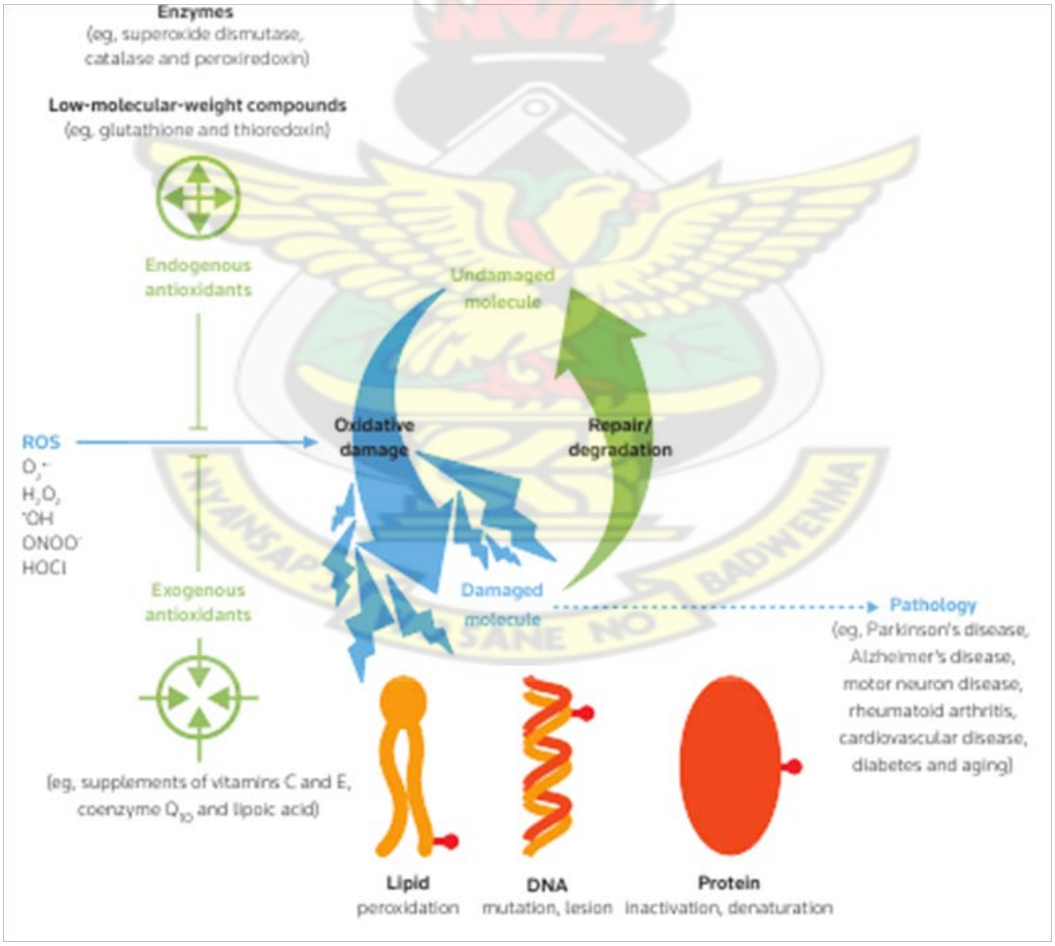


Figure 3.1: Antioxidants against Oxidative Damage (Rodriguez-Cuenca *et al.,* 2010).

Oxidative stress results in molecular damage to lipids, DNA and proteins, and can contribute to a range of pathologies. Antioxidants, both endogenous and exogenous, can protect this oxidative damage by detoxifying ROS. An ideal exogenous antioxidant should combine the following properties to function effectively as a therapeutic agent; (i) rational design to optimize the specificity and efficiency of the chemical reaction with its target ROS (ii) high bioavailability and uptake (iii) targeting within tissues to the subcellular site of ROS production and (iv) recycling of the inactive antioxidant back to its active form by endogenous pathways following detoxification of the ROS. Accurate biomarker assay should be available to measure the extend of oxidative damage and to confirm that the antioxidant is protective.

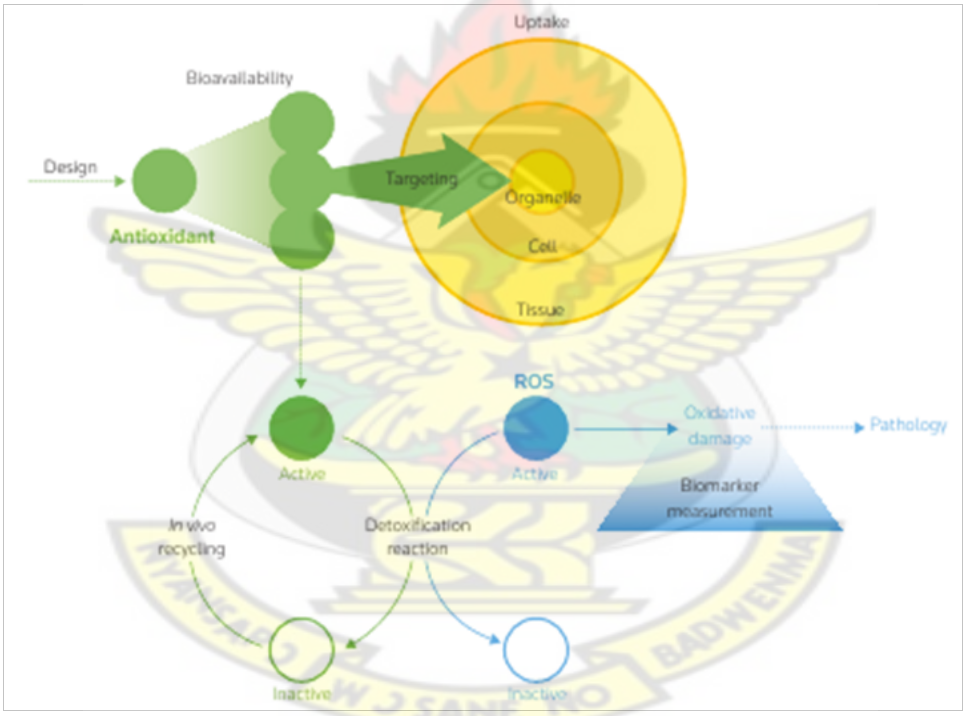


Figure 3.2: Properties of an Ideal Antioxidant (Rodriguez-Cuenca *et al.*,2010).

3.1.3 Inflammatory Mediation and Regulation of Ulcerative Colitis

Mediators of inflammation, respond to growth factors and cytokines released by lymphoid cells, and expresses integrins. Some chemokines, such as IL-8 and monocyte chemoatractant protein 1 (MCP-1), are inducible in nearly all cell types.

Interleukin-10 (IL-10) is a cytokine with potent anti-inflammatory and immune regulatory activity. IL-10 is produced by T cells, B cells, macrophages, thymic cells, and keratinocytes, and it down regulates the function of T helper (Th)-1 cells, NK cells, and macrophages (Hibi *et al.*,2002). It inhibits the production of inflammatory cytokines, such as IL1 and TNF-α, which stimulate production of ROS. IL-10 also inhibits production of reactive oxygen species in neutrophils and human monocytes (Narushima *et al.,* 2003).

IL-6 is associated with human colonic epithelium, and is secreted by freshly isolated colonocytes. IL-7 is a potent regulator of systemic and mucosal lymphocyte growth and differentiation. A role for IL-7 in the pathogenesis of UC is suggested by the high levels of IL-7 in the mucosa of ulcerative colitic patients (Gibson *et al.,* 1996). IL-2 is an indispensable regulatory cytokine of the immune system that has multiple functions, including the activation of T cells, macrophages, lymphokine-activated killer (LAK) cells, natural killer (NK) cells, the differentiation of B cells, and activation induced cell death (AICD). IL-15, a cytokine with effects similar to IL-2 and an inducer of tumour growth factor P (TGF-P), is synthesized and secreted by freshly isolated intestinal epithelia (Hibi *et al.,* 2002).

The histone deacetylase (HDAC) family of transcriptional co-repressors has been clearly demonstrated to regulate colonocyte maturation and subsequent transformation; HDAC inhibitors have been shown to induce growth arrest, differentiation, and apoptosis of cells derived from the GI tract (Jackson and Evers, 2009).

Platelet activating factor is a potent inflammatory factor that acts on neutrophils and monocytes. It is produced after calcium ionophore stimulation of freshly isolated colonocytes from patients with IBD. Eicosanoids; Leukotriene B4 (LTB4), is one of the most potent chemotactic and chemokinetic metabolites of arachidonic acid during intestinal inflammatory reactions like colitis. As a result, lowering of the LTB4 level could attenuate neutrophil activation and infiltration into the inflammed colonic tissue. Consequently free radicals generated by neutrophils and other granulocytes would also be markedly reduced (Ko and Cho, 2005). The colonic epithelium also generates a range of other eicosanoids, including thromboxane and prostaglandins (Gibson *et al.*, 1996).

Nitric oxide (NO) is an important regulator of gut inflammatory responses and plays an important role in the pathogenesis of ulcerative colitis (Tai *et al.*, 2007). Luminal overproduction of Nitric oxide, a vasoactive agent, induces hemorrhagic colitis and levels are increased in UC. Although infiltrating lamina propria cells are likely to be the major source of nitric oxide, intestinal epithelial cells also produce nitric oxide. This production is regulated by proinflammatory cytokines, interferon (IFN) and growth factors (Gibson *et al.*, 1996).

Exposure of innate immune cells, such as neutrophils, monocytes, macrophages, and mast cells, to glucocorticoids induces the release of annexin A1 (AnxA1), a downstream mediator of glucocorticoid signalling, shown to inhibit neutrophil adhesion to endothelial cells, decrease transmigration, promote the apoptosis of neutrophils, and promote macrophage-regulated phagocytosis. This could promote the resolution of the inflammatory reaction by actively maintaining homeostatic control of innate immune cells. Similarly, exposure of T cells to glucocorticoids leads to a reduction in annexin A1 expression and consequently inhibition of T cell activation and differentiation toward a Th 2-cell phenotype (Jackson and Evers, 2009).

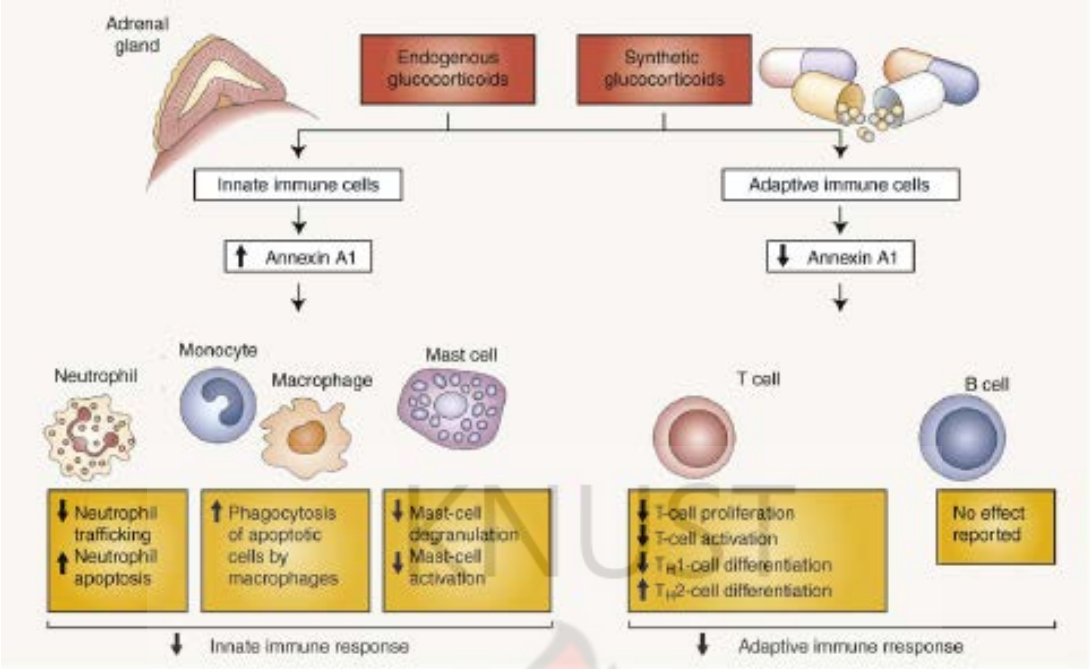


Figure 3.3: Glucocorticoid modulation of Annexin A1(Jackson *et al.*,2011).

An up regulation of TNF-related apoptosis-inducing ligand (TRAIL), a membrane-bound protein with homology to TNF-α, has been identified on enterocytes isolated from actively inflammed mucosa of patients with UC. Treatment of intestinal cells *in vitro* with the compound human beta defensin 2 (hBD-2) has led to promotion of intestinal wound healing by ameliorating TRAIL-induced apoptosis (Jackson and Evers, 2009).

Toll-like receptors (TLR) are members of a conserved IL-1 family of receptors that signal through NF-kB and mitogen-activated protein kinase (MAPK). Human intestinal epithelial cells normally express TLR3 and 5, with levels of TLR2 and 4 barely detectable. One school of thought reported that deletion of TLR5 in mice leads to the spontaneous development of colitis. Interestingly, another identified a peptide sharing homology with flagellin and that treatment with this antibody led to monocyte activation and modulation of proinflammatory genes upon binding with TLR5 (Jackson and Evers, 2009).

N-acetyl-cysteine (NAC) has been widely used as an antioxidant to diminish various parameters of colonic inflammation. It is known to act by raising intracellular concentrations of cysteine and hence of reduced glutathione (GSH) or by scavenging ROS. NAC is found to be a powerful scavenger of HOCl. It also increases the level of GSH in the mucosa, therefore, contributing to its beneficial effect in colitis (Nosal'ova *et al.*, 2000). Generally, prostaglandins (PGs) produced via COX-2, the inducible isoform of cyclooxygenase, are involved in both inflammation and regeneration whereas PGs derived from COX-1, the endogenous isoform, exerts immunomodulatory, cytoprotective, and pro-angiogenic effects (Hamada *et al.*, 2010)

3.1.4 Influential Factors of Ulcerative Colitis

Non-steroidal anti-inflammatory drugs, antibiotics, enteric infections, and stress have all been reported to be potential triggers of ulcerative colitis. Although the mechanisms can be hypothesized, their association with flares remains confusing (Felder *et al.*, 2000). .

3.1.4.1 The Role of Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs have been shown to specifically cause ulceration and strictures of the jejunum, ileum, and colon. The clinical manifestations of the large bowel tract toxicity is extensive and include diarrhoea, abdominal pain, blood and protein loss, colitis, and perforation (Felder *et al.*, 2000).

3.1.4.2 The Role of Antibiotics

An important connection between antibiotic use and exacerbation of symptoms is through the development of *Clostridium difficile* infections. It is widely believed that antibiotic use alters intestinal microflora, which may play a role in the pathogenesis of UC. *C. difficile* colitis is one example where antibiotics alter normal intestinal microflora infection (Singh *et al.*, 2009).

3.1.4.3 The Role of Normal Flora

Strong evidence for the role of normal enteric bacteria in intestinal inflammation is provided by the mitigation of the disease in animals bred in a germ-free environment, partly because bacteria and their products can stimulate inflammatory responses. Cell wall fragments of some bacteria elicit a response of chronic inflammation, when injected into the bowel of susceptible rats. In early stages of mucosal breakdown, colonic defence against luminal bacteria is impaired, and bacterial invasion contributes to tissue destruction (Videla *et al.,* 1997).

3.1.4.4 The Role of Stress

Stress is deemed as a threat to homeostasis. When a stress response is triggered, there is a cascading neuro-endocrine involvement with changing levels of sympathetic nervous system mediators. It can modulate the release of pro-inflammatory agents like neuropeptides such as substance P, altering the activity of immune and inflammatory cells. Symptoms such as pain and diarrhoea can be exacerbated by stress, but the increased symptoms do not necessarily reflect disease worsening in terms of inflammation.

The evidence to date does not support NSAID use, antibiotics, prior infections, or antecedent stress as initiating causes. However, there is compelling evidence of their role in the disease (Singh and Alter, 2004).

3.1.4.5 The Role of Alcohol

Alcohol is an ingredient common to wine, beer, and liquor. Its effect on any given system has been found to depend on the dose and type of alcoholic beverage. The rectal mucosa of chronic alcoholics has been shown to exhibit an increased mononuclear cell infiltrate, with more mitochondria and a hypertrophic endoplasmic reticulum, which disappear with abstinence.

The most frequent intestinal disorders observed among alcoholics are diarrhoea and malabsorption caused by alterations in the digestion and absorption of food. The intestinal permeability in chronic alcoholics increases, suggesting an increased endotoxemias. At low concentrations of about 5% alcohol, acid secretion is stimulated, whereas higher doses either exert no effect or showed inhibitory action, associated with mediation via the cholinergic system, topical stimulation of the parietal cells with an increase in cyclic adenosine monophosphate (cAMP) production, and histamine release (Bujanda, 2000).

The administration of a low alcohol dose accelerates gastric emptying, whereas high doses delay emptying and reduce bowel motility, due to toxic action of alcohol on the contractile proteins. Alcoholics present increased cortisol and corticotropin levels, which would act by inhibiting the musculature of the gastrointestinal tract. Ethanol and its metabolites, acetaldehyde, promote tumour development by generating free radicals and other oxidizing agents. Moreover, alcohol is directly cytotoxic and is able to produce aberrant methylation of DNA, with an impairment of its self-repair capacity (Bujanda, 2000). However, the polyphenols contained in wine, have important anticancer actions mediated by: Inhibition of COX and lipoxygenase, Enzyme modulation of xenobiotic metabolism, Catechin inhibition of cell proliferation, interrupting tumour promoters and their receptors, Inhibition of nitrosation reactions and Antioxidant activities which involves:

- The catechol chelation of active metals needed for the generation of free radicals.
- Catechin elimination of H_2O_2 and $^{\cdot}OH$, which damage DNA and initiate lipid peroxidation.
- Flavonols reaction with peroxy radicals, thus interrupting the lipid peroxidation chain

The bactericidal effect of wine is superior to that of solutions on *Salmonella* and *Shigella* enteritis, and similar to that afforded by bismuth salicylate against *E. coli* (Bujanda, 2000).

3.1.4.6 The Role of Tobacco

Epidemiologic studies have shown that smoke protects against the development of UC and controlled clinical trials have demonstrated that transdermal nicotine is efficacious for active UC. The risk of developing UC was found to be greater in both ex-smokers and non-smokers. Non-filtered and filtered tobacco smoke reduced the severity of colonic inflammation and lesion, because the anti-inflammatory action of tobacco smoke involved reduction of the elevated colonic MPO activity, prevention of neutrophil activation and their infiltration into the inflammed tissues (Ko and Cho, 2005).

Other protective mechanisms of nicotine on UC include the boosting of mucin synthesis on colonic luminal surface, release of NO that counteracts with eicosanoid metabolism, and the increase in intestinal trefoil factors (ITFs) that eventually improve mucosal restitution (Wu and Cho, 2004). Cigarette filter can separate the tar and gas phases of tobacco smoke with most of the particles in the tar phase, including ROS, hydrocarbons, and phenols being retained in the filter (Ko and Cho, 2005). These extracted compounds from the tar phase of tobacco smoke could repress mucus synthesis by inhibiting polyamine synthesis and thus potentiate ethanol-induced gastric mucosal injury (Ma *et al.*, 2000). Although the gas phase also contains radicals, these organic radicals usually have a shorter half-life of less than 1 sec. Hence, filtered gas could produce prominent antioxidative effects that contribute to colonic protection in a similar manner to the action of systemic nicotine treatment (Ko and Cho, 2005). When the damaging factors are filtered, the net effect could be colonic protection as evident below.

- Approximately 2/3 of former smokers develop the disease after smoke cessation
- There is UC onset after smoke cessation and UC remission with smoke resumption
- Remission of active UC in patients who have never smoked but who began smoking
- A flare of UC in remission after smoke cessation

3.1.5 Response to Injury

The response of the intestinal epithelium to injury is complex and involves many phenotypic, secretory, and biochemical changes. Following intestinal epithelial injury, cells from adjacent undamaged sites flatten and rapidly migrate to cover the exposed stroma, a process termed restitution. This response begins within minutes after injury and spans over an hour, obviating cell proliferation. Regeneration of the epithelium involves the replacement of both lost cells by accelerating proliferation and lost crypts by a single crypts becoming larger and then bifurcating from the base up-ward (Gibson *et al.*, 1996).

Cells with the capability of secreting epithelia growth factor (EGF) and trefoil peptides develop in association with budding crypts. The colonic epithelium is capable of acetylation, sulphation of phenols, hydroxylation, and S-methylation, but not of glucuronidation. Butyrate plays a key role in xenobiotic metabolism by the supply of acetyl CoA (Gibson *et al.*, 1996).

In UC, there is likelihood of poor supply, reduced uptake, and impaired use of glutamine by enterocytes with potential consequences of impaired repair and barrier function and increased susceptibility to injury. Similarly, endotoxemia or sepsis are associated with reduction of uptake and metabolism of glutamine, mimicking the fasting state. Epithelia growth factor (EGF) stimulates glutamine uptake by enterocytes, and this may have relevance in the setting of epithelial restitution and regeneration. The intestinal epithelium is able to secrete cytokines and other mediators of inflammation, responds to growth factors and cytokines released by lymphoid cells, and expresses integrins and HLA class II molecules, permitting direct cell-cell communication with inflammatory and immune cells. Both pro-and anti-inflammatory cytokines and IFNs have wide-ranging effects on intestinal epithelium (Gibson *et al.*, 1996).

The trefoil factor family (TFF) is a relatively new family of peptides, which are expressed and secreted in a tissue-specific manner in the gastrointestinal tract. These factors protect epithelium from injury, promote repair through restitution and wound healing, and inhibit tumour growth (Yang *et al.*, 2005). In the gut, defensins and polypeptide antibiotics with wide spectra of action, secreted by paneth cells into the intestinal lumen, have potent activity against a range of enteric pathogens (Gibson *et al.*, 1996).

The cell proliferative response in healing is dependent on activation of ERKYBMP-1, a specific mitogen activated protein kinase 1 and the mediator STAT3 (signal transduction and activator of transcription 3), by inhibiting phospholipase A2 activity in arachidonic acid metabolism. Luminal EGF has been found to be active in regulating cell proliferation and function throughout the gastrointestinal tract. It inhibits gastric acid secretion, up-regulates intestinal electrolyte and nutrient transport, and induces expression of brush border enzymes such as disaccharidases (Beck and Podolsky, 1999).

The cytokines IL-6, IL-8, and TNF-α, for example, are crucial for immune cell survival and proliferation of neoplastic cells. Additionally, TNF-α signaling leads to the apoptosis of enterocytes and loss of mucosal integrity. The carbohydrate-binding protein galectins (gals) play an important immunoregulatory role in intestinal inflammation; while gal-1 and gal-2 have proven to contribute to the suppression of intestinal inflammation by inducing apoptosis of activated T cells, gal-4, which is expressed only within the digestive tract, has been shown to exacerbate inflammation via the stimulation of IL-6 production by CD4+ T cells (Evers *et al.*,2009; Jackson and Evers, 2009). Cytokines and toxic molecules produced by these cells are also a rich source of proteolytic enzymes such as the serine protease, neutrophil elastase, and various acidic cysteine proteases (MacDonald and Pender, 1998).

In addition, endogenous fibroblast-like stromal cells are a potent source of matrix metalloproteinase (MMPs), which can degrade the lamina propria in chronic gut inflammation. More so, proteinases produced by infiltrating inflammatory cells can cause tissue injury. The stromelysins are more important early in disease control, producing mucosal ulceration, and gelatinases may be important in remodeling the mucosa during healing, as in wound healing (MacDonald and Pender, 1998).

Because lumen-to-lamina-propria passage of macromolecules occurs by complexly regulatory processes, its repair mechanisms are highly efficient. It has potentially important interactions with the mucosal immune system, and its metabolic and secretory capabilities may play key protective roles independently of its function as a physical barrier (Beck and Podolsky, 1999). Inflammatory conditions of the GIT can result from inappropriate regulation of these broad functions. Barrier function requires regulation of cell proliferation and differentiation as well as secretion of cellular products into the luminal surface including mucus, acid, bicarbonate, trefoil peptides, and antibodies (Beck and Podolsky, 1999).

The major energy source for colonic epithelial cells is butyrate, which by virtue of its even number of carbon atoms, undergoes rapid P-oxidation to acetyl co-enzyme A. Butyrate, acetate and propionate, which do not undergo P-oxidation, are produced largely in the colonic lumen by bacterial fermentation of carbohydrates, principally fiber. Impairment of the barrier leads to exposure of the lamina propria to molecules with antigenic, toxic, immune-adjuvant and chemoatractant properties with subsequent responses from natural and immune-mediated defences. Mucosal immune effector mechanisms are predominantly directed towards luminal molecules indicating failure of epithelial protection (Beck and Podolsky, 1999).

3.1.6 Other Types of Colitis

3.1.6.1 Ischemic Colitis

Ischemic colitis is a definite disease control entity that proceeds in 3 conditions; transient, stricture, and gangrenous. Today, ischemic colitis is considered to be a common disease of the large intestine, and is thought to be caused by various factors including vascular factors such as ischemia and embolism, as well as intestinal factors such as constipation, irritable bowel syndrome, and history of intestinal surgery (Matsumoto *et al.*, 2008). The condition is characterized by diffuse haemorrhage and oedema in the submucosal layer, degeneration, desquamation and necrosis of the mucosal epithelium, congestion of the lamina propria, thrombi in the capillaries, and slight neutrophilic infiltration (Matsumoto *et al.*,2008).

3.1.6.2 Non-Steroid Anti-Inflammatory Drug (NSAID) Induced Colitis

A diverse constellation of pathological findings have been reported in NSAID-induced colitis. These include mucosal ulceration, collagenous colitis, non-gangrenous ischemic colitis, and focal active colitis resembling Crohn's disease or infectious colitis. It is postulated that NSAIDs open paracellular pathways by inhibition of prostaglandin, allowing access of luminal contents to the lamina propria. This might induce inflammation and ulceration as well as activate the pericryptal fibroblasts, leading to thickening of the collagen table (Kakar *et al.*, 2003).

An increase in endothelial permeability might also allow translocation of endogenous flora and play a role in sustained inflammation and injury to the colon. The cornerstone of therapy in NSAID-induced colitis is discontinuation of the drug (Kakar *et al.*, 2003).

3.1.6.3 Antibiotic Induced Colitis

Recent research interest in anaerobic infections has waned to some extent. But following large populations of immunosuppressed patients, serious opportunistic infections are resulting in a re-emergence of anaerobes as an important component in the world of clinical infections. There is significance of an increase incidence of pseudomembranous colitis in human patients treated with clindamycin and the isolation of *Clostridium difficile,* complemented with the increasing antibiotic resistance among clinically significant anaerobes (Onderdonk, 2005).

3.1.6.4 Microscopic Colitis

This speaks of inflammation of the colon whose lining appears normal even under colonoscopy. The diagnosis of microscopic colitis is made only by taking biopsies of the Pseudo-normal-colonic lining, and then examining the biopsies under a microscope. There are two types of microscopic colitis: collagenous and lymphocytic colitis (Medicine Net, 2011).

The collagenous colitis is characterised by chronic, non-bloody, watery diarrhoea and an increase in the subepithelial collagen table, intraepithelial lymphocytosis, and an increase in lamina propria chronic inflammation. The cause is unknown and it does not appear to be contagious, but is sometimes hereditary. There can be an association with other auto-immune disorders, such as thyroid disorders, diabetes and rheumatoid arthritis (Kakar *et al.*, 2003).

In lymphocytic colitis, there is an accumulation of lymphocytes within the lining of the colon. Some experts believe that lymphocytic colitis and collagenous colitis represent different stages of the same disease. The inflammation and the collagen probably interfere with absorption of water from the colon, and cause the diarrhoea (Medicine Net, 2011).

3.1.7 Therapies of Ulcerative Colitis

3.1.7.1 Chemotherapy

When tissue influx of polymorphic nuclear cells (PMN) and macrophages occurs, a marked increase in the production of reactive oxygen metabolites (ROM) and leukotrienes (LT) will result as the secondary amplification of the inflammatory responses. For this reason, new therapeutic agents are been soughed for the treatment of UC. Of the existing three classes of medications; Aminosalicylates, Glucocorticoids and Immunomodulators, the aminosalicylates (sulfasalazine, olsalazine, balsalazide, oral and rectal mesalamine) are the standard first line therapies for UC, and patients who fail to respond to these agents are usually treated with oral glucocorticoids (Lehne *et al.,* 2004).

The aminosalicylates are used to treat mild to moderate ulcerative colitis and to maintain remission after symptoms have subsided. The glucocorticoids as inhibitors of neutrophil formation and activation, capable of decreasing the margination of neutrophils, and inhibiting neutrophil aggregation (Ko and Cho, 2005), and eicosanoid metabolism are indicated primarily for induction of remission and long-term-maintenance, while the immunomodulators are employed for moderate to severe disease situations. But none is curative, at best, remission and maintenance (Lehne *et al*., 2004).

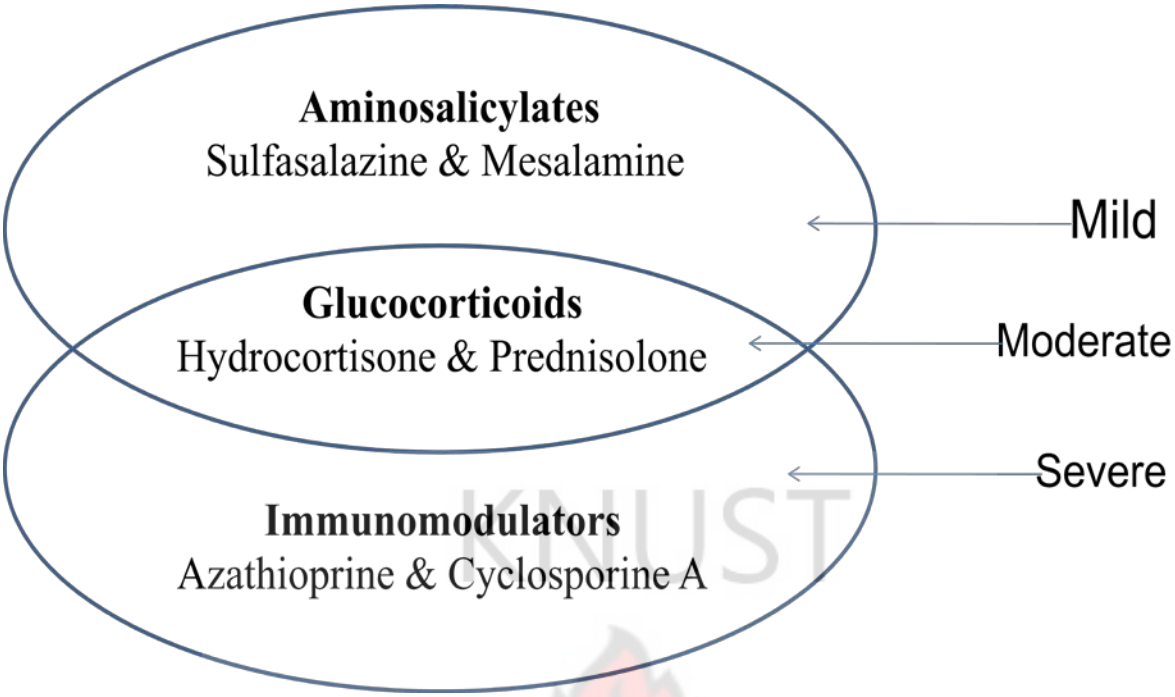


Figure 3.4: Therapeutic Course of Ulcerative Colitis.

3.1.7.1.1 Five (5)-aminosa-licylic acid (5-ASA)

Preparations of aminosalicylates, exemplified by 5-aminosalicylic acid (5-ASA), for oral or colonic administration have been used over many decades for the treatment of the inflammatory bowel disease, ulcerative colitis and Crohn's disease (van Bodegraven and Mulder, 2006). 5-ASA, known as mesalamine or mesalazine, is also considered to be the active therapeutic moiety of the other classical anti-colitic agent, sulphasalazine (Horvath *et al.*, 2008). Its complex pharmacological profile includes inhibition of intestinal macrophage chemotaxis and mononuclear cell antibody secretion (Horvath *et al.*, 2008), inhibition of pro-inflammatory cytokine release and inhibition of the arachidonate lipoxygenase and cycloxygenase pathways. More recent molecular studies have identified actions on the nuclear factor, NFκB, and on the peroxisome proliferator-activated receptor-γ (PPAR-γ), a nuclear receptor (Rousseaux *et al.*, 2005).

However, an enduring concept is that, at least part of the beneficial activity of 5-ASA reflects its actions as an antioxidant and free radical scavenger (Reifen *et al.,* 2004). Thus, the generation and release of local reactive oxygen species have long been considered to be involved in the vascular, epithelial and mucosal inflammatory injury and hence scavenging these moieties offers a potential mechanism of action of 5-ASA (Dryden *et al.*, 2005).

3.1.7.1.2 Selective Cox-2 Inhibitor

The discovery of cyclo-oxygenase (Cox) isoforms had had a significant impact on the understanding and management of inflammatory diseases. Cox-1 and Cox-2 catalyze the synthesis of prostaglandins. Cox-1 is expressed in most mammalian tissues, and prostaglandins produced by Cox-1 are thought to play a major role in the maintenance of gastrointestinal homeostasis. In the colon Cox-1 is expressed in areas of proliferation in the lower crypt, but its expression is lost as the epithelial cells differentiate and migrate higher up the crypt. Cox-2 on the other hand is induced in monocytes and macrophages by proinflammatory cytokines, mitogens, and endotoxin within a few hours of exposure to invasive organisms and then is lost over a period of approximately 24 h (El Miedany *et al.*, 2006).

There is evidence that COX-2 inhibitors cause fewer gastrointestinal complications than with nonselective NSAIDs and attenuates the development of colonic polyps. COX-2 inhibitors are as good as the combination of nonselective NSAIDs and a proton pump inhibitor (PPI) in patients at risk of ulcer complications (Chan, 2008). In UC, Cox-2 is only expressed in non-proliferating cells and is induced in cells at the level of the mid crypt. Cox-2 inhibitors have better safety profile than traditional NSAIDs in patients with inflammatory bowel disease. Etoricoxib is an example of Cox-2 selective agent (El Miedany *et al.,* 2006).

3.1.7.1.3 Glucocorticoids

Glucocorticoids (GCs) have become the mainstay of medical treatment, but 20-30% of patients with UC respond poorly, or not at all, to GCs treatment. The beneficial effects of conventional GCs in UC are often offset by troublesome systemic side effects like acne, moonface, striae, hypertension, dyspepsia, impaired glucose tolerance, and mood disturbances, including insomnia. Long-term treatment (> 6 months) with GCs like prednisolone in doses higher than 7.5-10 mg daily, is precluded by the risk for certain hazardous and irreversible complications such as osteoporosis and osteonecrosis, cataract, and overt diabetes mellitus (Lofberg, 1995).

The new generation GCs, such as budesonide and fluticasone propionate, have a very high receptor affinity. Thus they exert a potent GCs action when given, but both are rapidly and extensively metabolized via the cytochrome P-450 when traversing the liver. In distal UC and proctitis, delivery can be achieved using conventional retention enemas or foam/gel preparations (Lofberg, 1995).

3.1.7.1.4 Immunomodulators

This class of medications including Azathioprine, Mercaptopurine, Cyclosporine, Methotrexate and Infliximab are capable of inducing and maintaining remission in UC, but the onset of their pharmacological effect could be delayed for up to 6 months. Thus they are not employed for acute monotherapy. These drugs are also found to be more toxic than the aminosalicylates and glucocorticoids, manifesting generalized immune-suppression and so they are mostly treated as last resort for patients who do not respond to the traditional therapy (Lehne *et al.*, 2004).

3.1.7.2 Phytotherapy

Phytogenic agents have traditionally been used by herbalists for the treatment of ulcers. Ethnopharmacological studies observed that the first drug effective against gastric ulcer, carbenoxolone, was discovered from *Glycyrrhiza glabra*, a commonly used indigenous plant. Also, studies on cabbage, previously employed as an anti-ulcer agent in folk medicine, has led to the development of gefarnate (Borrelli and Izzo, 2000). Thus, a search among medicinal plants is still important (Borrelli and Izzo, 2000).

3.1.7.2.1 Flavonoids

Flavonoids, a group of about 4000 naturally occurring compounds with a wide range of biological effects, including anti-ulcer activity, have been proposed to increase mucosal prostaglandin content and decrease histamine secretion from mast cells by inhibition of histidine decarboxylase. In addition, flavonoids have been found to be free radical scavengers. Other possible mechanisms include inhibition of the gastric proton pump, inhibition of the lipoxygenase, pathway inhibition of platelet activating factor synthesis, inhibition of lipid peroxidation and scavenging of free radicals associated with a significant enhancement in glutathione peroxidase activity (Borrelli and Izzo, 2000).

3.1.7.2.2 Naringin

Naringin has been shown to prevent gastric mucosal ulceration in several animal models. It significantly reduces the ulcer index and increases the hexosamine content of mucus without affecting prostaglandin E2 and the total protein content. Thus, the gastroprotective action of naringin could be explained, at least in part, as a non-prostaglandin-dependent mechanism that involves an increase in glycoprotein content and viscosity of the gastric mucosa. It also has an antioxidant property which could be associated with gastroprotection (Borrelli and Izzo, 2000).

3.1.7.2.3 Anthocyanosides

Anthocyanosides are a significant preventive and curative anti-ulcer where there are lesions. The anti-ulcer activity is not exerted through a blockade of gastric secretion but the increase in the mucus. They are thought to act by influencing the biosynthesis of the mucopolysaccharides, thus improving the efficiency of the mucus barrier (Borrelli and Izzo, 2000).

3.1.7.2.4 Saponins

Saponins have haemolytic properties and are highly toxic when injected into the blood stream. When taken by mouth saponins are comparatively harmless. The protective activity of saponins is not due to inhibition of gastric acid secretion but probably due to activation of mucous membrane protective factors (Borrelli and Izzo, 2000).

3.1.7.2.5 Tannins

Tannins are known to 'tan' the outermost layer of the mucosa and to render it less permeable and more resistant to chemical and mechanical injury or irritation. When a low concentration of tannins is applied to the mucosa, only the outermost layer is tanned, becoming less permeable and affording an increased protection to the subjacent layers against the action of bacteria, chemical irritation and to a certain extent, against mechanical irritation. High concentrations of tannins cause coagulation of the proteins of the deeper layer of the mucosa, resulting in inflammation, diarrhoea and vomiting (Borrelli and Izzo, 2000).

The anti-ulcerative effect of silymarin could be related to its inhibitory mechanism on the lipoxygenase pathway, avoiding leukotriene synthesis (Borrelli and Izzo, 2000).

3.1.8 Challenges in Ulcerative Colitis' Drug Development

The challenge of developing a potential new medicine without clinical intestinal liability is not only hampered by biological complexity of the system under scrutiny, but also by fundamental differences in terms of GI function between the rat, the most commonly used experimental animal and man. For example, the rat GI tract as a system probably best studied *in vivo*, has no gall bladder and does not vomit (Safety Medicines Report, 2005).

3.1.9 Experimental Models of Ulcerative Colitis

There are experimental models with a variable range of clinical manifestations similar to those observed in human ulcerative colitis. These models contributed greatly to important advances in our current understanding of the underlying mechanisms of inflammation and disease pathogenesis as well as treatment (Jurjus *et al.,* 2004). In general, an appropriate animal model should display such key characteristics as; the GIT morphological alterations, inflammatory signs and symptoms as well as pathophysiology similar or identical to the human UC.

Six broad classes of such animal models are reviewed herein (Jurjus *et al.,* 2004).

1. Spontaneous colitis models
2. Gene knockout (KO) models
3. Transgenic mouse and rat models
4. Adoptive transfer models
5. Inducible colitis models
6. Less frequent microbial models

3.1.9.1 Spontaneous colitis models

3.1.9.1.1 C3H/HejBir mice

C3H/HejBir is a derivative of selective breeding of C3H/Hej mice with colitis known to develop perianally. It occurs spontaneously in the 3rd – 4th week and disappears after 10–12 weeks. Ulcers and crypt abscesses are seen, but thickening of the intestinal wall and granulomas are not observed. Increased levels of IFNγ and IL-2 have been detected in the lamina propria lymphocytes, suggesting a Th type-1 response (Jurjus *et al.*, 2004).

3.1.9.1.2 SAMP/Yit mice

SAMP/Yit mice are a substrain obtained by selective breeding of AKR mice. Colitis develops in all mice by the 30 th week, with all-layer-skip lesions, and crypt abscesses. Laminar propria lymphocytes, when stimulated will generate higher levels of IFNγ and TNFα than those of AKR mice. Antibody blockade of the intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, ameliorated inflammation in the SAMP/Yit. Thus, the blocking of ICAM-1 and VCAM-1 may have a therapeutic benefit (Hibi *et al.*, 2002).

3.1.9.2 Gene knockout (KO) models

3.1.9.2.1 Interleukin-2 KO/IL-2 receptor (R)α KO mice

In mice with a disrupted IL-2 gene, the small intestine remains intact, whereas the colon (from rectum to caecum) is severely affected with ulcers and wall thickening. Pathologically, crypt abscesses, mucin depletion, and dysplasia of the epithelial cells as well as infiltration of activated T cells and B cells are observed. Prolonged inflammation in IL-2 KO mice is presumed to be due to the impairment of acquired immune combined deficiency (AICD). IL-2-dependent T cells are generated in the thymus at an early stage after birth, a deficit of these regulatory T cells induces a Th type-1 response in this model (Hibi *et al.*, 2002).

3.1.9.2.2 IL-10 KO mice models

IL-10 produced by T cells, B cells, macrophages, thymic cells, and keratinocytes, down regulates the function of T helper (Th)-1 cells, NK cells, and macrophages (Jurjus *et al.,* 2004). Inflammation occurs in the whole intestine of IL-10 -/- mice. In the colon, goblet cell depletion, degeneration of the epithelium, infiltration of IgA-producing plasma cells, and an increase in MHC class II expression are detected. As in the IL-2 -/- mice, the activation of CD4 + Th1 cells and the depletion of their inhibitor, the regulatory T cells, are presumed to be the cause of the inflammation (Jurjus *et al.,*2004). Colitis that developed in the IL-10 -/- mice evolved into two distinct phases: IL-12 played a pivotal role in early colitis, whereas its absence and the synthesis of IL-4 and IL-13 in late disease indicated that other immune mechanisms sustained chronic inflammation. Thus, it is suggested that impairment of the regulation of macrophages, but not of T cells, was of much significance (Hibi *et al.,* 2002).

3.1.9.2.3 T cell receptor (TCR) α mutant mice

While most animal models have Th type-1 immune responses (IFNγ and TNF-α predominant), this model is suggested to have a Th type-2 immune response (IL-4, IL-5-predominant) due to a decrease in the binding affinity between TCR and MHC, due to the depletion of the α chain, and the secretion of IL-4 (Hibi *et al.,* 2002). Soft stools, persistent inflammation, and hypertrophy of the colon are observed in 16 weeks after birth, but the small intestine remains intact (Jurjus *et al.,* 2004). Hyperplasia of the colonic epithelium, a decrease in the number of crypt abscesses and goblet cells, and infiltration of lymphocytes, plasma cells, and neutrophils are also obvious. It is presumed that only in the TCRα -/- mice are colitis permanent. Auto-antibodies are produced as a result of the immunological disorder and presumed to work against, rather than promote the inflammation (Hibi *et al.,* 2002).

3.1.9.2.4 TNF-3' Untranslated Region (UTR) KO Mice

Colitis would occur in mice with over expression of human TNFα. There is an adenine/uracil-rich element (ARE) consisting of AUUUA repeats in the 3V-UTR area of IL-2, c-fos and granulocyte macrophage–colony-stimulating factor (GM–CSF) which destabilizes the mRNA of the cytokines in the upstream region (Kontoyiannis *et al.,* 1999). Mice deficient in the ARE of TNFα show high levels of serum TNFα and have arthritis and colitis (Hibi *et al.,* 2002).

3.1.9.2.5 Trefoil Factor-Deficient Mice

Intestinal trefoil factors (ITFs) are peptides secreted by mucus cells of the intestine after inflammatory damage. Mice with disrupted ITF shows severely impaired mucosal healing and decreased epithelial regeneration (Jurjus *et al.*, 2004).

3.1.9.3 Transgenic Mouse and Rat Models

3.1.9.3.1 IL-7 Transgenic Mice

It has been demonstrated that IL-7 within the serum of UC patients influences the differentiation and proliferation of T cells in the thymus (Hibi *et al.*, 2002). Further investigation of IL-7 transgenic mice, revealed that acute colitis occurred in 1 to 3 weeks of age along with infiltration of neutrophils, CD4 + T cells, and T cells in the intestine followed by proctoptosis with anal bleeding at 8–12 weeks of age. This is therefore a chronic colitis model that closely resembles human UC (Jurjus *et al.*, 2004). It is suggested that, in the acute phase, the excessive secretion of IL-7 induces activation of the mucosal lymphocytes to causes colitis, while in the chronic phase, apoptosis of the activated lymphocytes which results from the lack of IL-7, is presumed to be the cause (Hibi *et al.*, 2002).

3.1.9.3.2 (STAT)-4 Transgenic Mice

Each of the seven Signal Transduction and Activator of Transcription (STAT) family works for several cytokines. STAT-4 is peculiar to the signal transduction of IL-12, and STAT-4 -/- mice are similar to KO model of Th type-1 colitis (Jurjus *et al.*, 2004).

3.1.9.3.3 HLA-B27 Transgenic Rats

A rat transgenic for human HLA-B27 and h2-micro-globulin develops spontaneous IBD that affects the stomach, ileum, and the entire colon. Crypt hyperplasia and mucosal infiltration mostly characterizes the disease (Jurjus *et al.*, 2004). This model has been used extensively to demonstrate that various bacterial species can induce diverse types of pathology in colitis and gastritis (Rath *et al.*, 2001). Increased levels of IFNγ and IL-2 in the lamina propria lymphocytes, suggest a Th type-1 response (Hibi *et al.*, 2002).

3.1.9.4 Adoptive Transfer Models

3.1.9.4.1 Heat Shock Protein (Hsp) Transfer Colitis (60-Specific CD8 T Cells)

Colitis in these mice required the presentation of hsp60 on MHC class I and depend on a functional role of TNFα. Initial analysis of this model indicates that an autoimmune hsp60 CD8 + T cells reactive to cellular hsp60, mediated the pathogenesis of colitis (Jurjus *et al.*, 2004).

3.1.9.4.2 CD45RB Transfer Model.

The adoptive transfer of CD4 + T cells expressing high levels of the surface molecule D45RB (CD4 +CD45RBhi) into severe combined immune deficient (SCID) recipients (i.e., CD4 + CD45RBhi T cells) will result in chronic non-bloody diarrhoea and wasting. The disease is progressive un-remittive and deadly. A histopathologic changes are similar to other models of colitis, and is limited to the colon, which is markedly hyperplasiac (Jurjus *et al.*, 2004).

3.1.9.5 Inducible Colitis Models

3.1.9.5.1 Trinitrobenzene Sulfonic Acid (TNBS) Induced Colitis

Colitis would occur in mice by treatment with a TNBS enema after destruction of the mucosal barrier with an ethanol enema (Hibi *et al.,* 2002). Susceptibility to TNBS varies in each mouse, but some will develop hapten-induced delayed-type hypersensitivity and proceeds to develop chronic colitis. Granulomas with infiltration of inflammatory cells in all layers are obvious. Isolated macrophages will produce large amounts of IL-12, and the lymphocytes large amounts of IFNγ and IL-2. This evidence suggeste that the colitis in this model is of a Th type-1 response (Jurjus *et al.,* 2004).

3.1.9.5.2 Oxazolone Induced Colitis

Comparing with TNBS, oxazolone causes colitis earlier. Body weight loss and diarrhoea is seen on the second day after the enema, and symptoms diminish after 10–12 days with the ulcers localized in the distal colon. Histopathological studies show that the numbers of epithelial, goblet, and glandular cells decreases, compared with controls (Jurjus *et al.,* 2004).

3.1.9.5.3 Iodoacetamide Induced Colitis

This model is based on the fact that the, instillation of sulfhydryl (SH) blocker in the colon could induce colitis by decreasing the amount of defensive SH compounds. After the induction of UC, diarrhoea, dilatation, adhesion, mucosal damage and inhibition of body weight gain determines the degree or severity of the disease control (Jurjus *et al.,* 2004).

3.1.9.5.4 Dextran Sulphate Sodium (DSS) Induced Colitis

The administration of DSS contained in water causes haematochezia, body weight loss, shortening of the intestine, mucosal ulcers and neutrophil infiltration. The acute colitis is considered to be induced by innate immunity but not acquired immunity because it also occurs in SCID rats. The Chronic phase on the other hand is said to be caused by lymphocytes that are activated by the cytokines secreted from the activated macrophages (Jurjus *et al.,* 2004).

3.1.9.5.5 Indomethacin Induced Enterocolitis

It has been shown that indomethacin induces small intestinal and colonic ulceration in a dose-dependent fashion in rodents (Elson *et al.,* 1995). Initial epithelial damage is mediated partly by inhibition of the protective prostaglandins PGE1, PGE2, and prostacyclin. Luminal bacteria and bacterial products clearly contribute to the inflammatory response (Jurjus *et al.,* 2004).

3.1.9.5.6 Acetic-Acid-Induced Colitis

The initial injury in this disease model is a relatively bland epithelial necrosis and oedema that variably extends into the lamina propria, submucosa, or external muscle layers. The epithelial injury is a specific reaction to organic acids, because HCl at similar pH wound not induce similar injuries (Jurjus *et al.,* 2004). Mucosa inflammation follows initial injury and is associated with activation of arachidonic acid pathways. This is an easily inducible model of UC, and the similarity of the inflammatory mediators suggests that the inflammatory phase bears semblance to acute human intestinal inflammation (Elson *et al.,* 1995). Treatment with an antioxidant improves the macroscopic and microscopic scores (Choudhary *et al.,* 2001).

3.1.9.6 Less Frequent Models

3.1.9.6.1 Peptidoglycan–Polysaccharide (PG–PS) Colitis

The intramural injection of bacterial cell wall component PG–PS into the colon of rats induces enterocolitis with thickening of the colon, infiltration of macrophages, and neutrophils. PG–PS increases mucosal permeability and MPO activity, and enhances nitric-oxide production and collagen synthesis. This model clearly shows that the cell wall components of nonpathogenic resident enteric bacteria are sufficient to induce colitis (Jurjus *et al.*, 2004).

3.1.9.6.2 Germ-Free Mice Colitis

The germ-free mice model exhibits some characteristics similar to human UC when microbial isolates from the faeces of genetically identical mice are introduced (Jurjus *et al.*, 2004). Colitis occurs in the germ-rich colon under specific pathogen-free conditions, but not under germ-free conditions. At least, its inflammatory mechanisms are thought to be the dysregulation of inducted tolerance to intestinal antigens, due to impaired immune regulation (Hibi *et al.*, 2002).

3.1.9.6.3 Radiation Induced Colitis

This is a relatively novel model. A combination of gamma irradiation and MHC class II deficiency in mice results in 100% penetrance of colitis.

Interestingly, it appears to be the case that the proliferation of animal models of UC has not only fuelled the detection of novel therapeutic agents but has also contributed to the understanding of the pathogenesis of UC (Jurjus *et al.*, 2004).

3.2 MATERIALS AND METHOD

3.2.1 Drugs and Chemical Reagents

Indomethacin, Glacial Acetic acid, tween 80, potassium phosphate buffer tablet, cetrimide, O-dianisidine, Pentobarbitone Thiobarbituric acid and Trichloro-acetic acid were supplied by Sigma-Aldrich Inc., St. Louis, MO, USA. Diethyl Ether, Petroleum ether, Ethyl-Acetate, Ethanol, chloroform, xylene, haematoxylin, eosin and DPX mountant were purchased from Gain land Chemical Company, Sandycroft, Deeside, UK. Thirty percent (30%) Hydrogen peroxide and sulphuric acid obtained from PS-Park Scientific Ltd, Northampton, UK. Prednisolone was acquired from Ernest Chemist, Accra, Ghana. Test drugs (*Trichilia* fractions) were constituted into 30 mg, 100 mg, and 300 mg.

3.2.2 Animals

Animals were obtained from the Animal House of the Department of Pharmacology, KNUST, maintained in an animal holding room at 24℃ ± 2℃ with a relative humidity of 70% ± 5˚c and a 12 h light-dark cycle. They were also fed on commercial pellet diet from Ghana Agro Food Company (GAFCO), Tema-Ghana, and provided water *ad libitum.* All animal experiments were carried out upon approval by the Faculty Ethics Committee and in compliance with stipulated international standards for laboratory animal use (NIH, 1996).

3.2.3 Experimental Groupings

Generally, Wistar rats of comparable age and weight (150 – 200g) were employed to guarantee the comparability and reproducibility of independent animal experiments. The experimental animals were housed five (5) per cage (34 x 47 x 18 cm) in six groups for each experimental set-up as in Fig. 3.5. All such animal cages had wood shavings as beddings otherwise wire bottom cages of same size and making were employed.

Of the six (6) groups;

- **GA** = Normal control group; fed with normal animal feed and tap water through the entire experimental period. (Naive)

- **GB** = Disease control group; treated with ulcerogenic agent then fed with normal animal feed and tap water for the rest of the experimental period. (Negative control)

- **GC** = Standard control group; treated with ulcerogenic agent then fed with normal animal feed alongside a standard drug for the experimental period (Positive control)

- **GD** = 30 mg/kg dose group; treated with ulcerogenic agent then fed with normal animal feed alongside TMBE for the experimental period. (Parallel treatment)

- **GE =** 100 mg/kg dose group; treated with ulcerogenic agent then fed with normal animal feed alongside TMBE for the experimental period. (Parallel treatment)

- **GF =** 300 mg/kg dose group; treated with ulcerogenic agent then fed with normal animal feed alongside TMBE for the experimental period. (Parallel treatment)

3.2.4 Work Plan

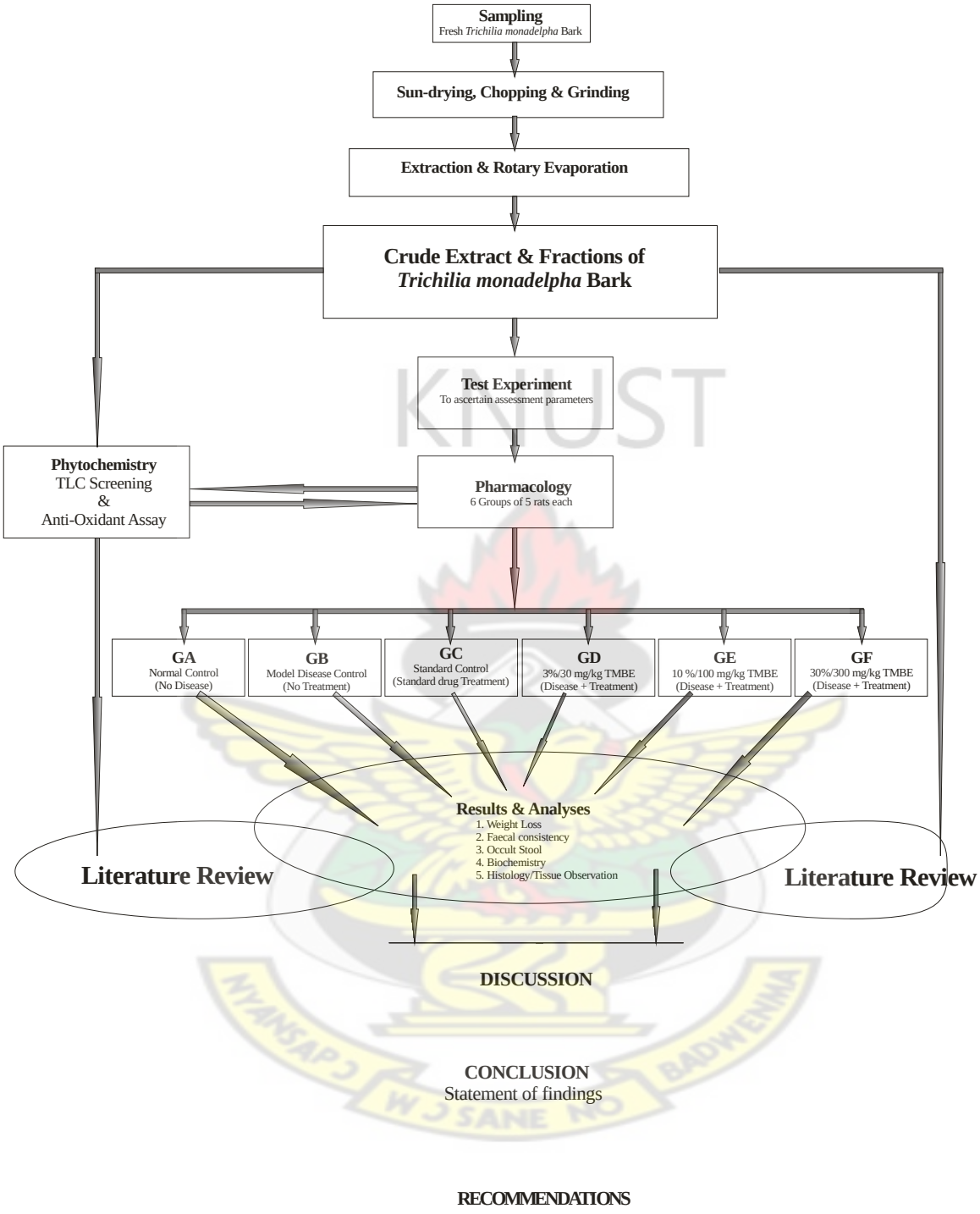


Figure 3.5: Conceptual Framework

3.2.5 Induction of Colitis

The chemically induced animal models produced lesions with clinical and pathologic features resembling human ulcerative colitis. For the chemically induced colitis, indomethacin and acetic acid were employed as ulcerogenic agents.

3.2.5.1 Indomethacin

Indomethacin suspension was prepared using Tween 80 and administered once using oral gavage (7·5 mg kg^{-1} body weight), to induce colonic ulceration in rodents. This model has the advantage of being easily induced, in acute or chronic phases (Elson *et al.,* 1995).

3.2.5.2 Acetic acid

In the acetic acid induced colitis, the experimental animals were slightly sedated by ether suffocation, following a 24 h fast, and then two millilitres (2 ml) of acetic acid (3% v/v in 0.9% saline) or saline alone (control animals) were infused using a gavage (external diameter 2 mm inserted into the anus and the tip advanced to 8 cm proximal to the anus). Acetic acid was then retained in the colon for 30 s after which the fluid was withdrawn (Jurjus *et al.*, 2004). Thus, induction of regulatory T cells does not occur, and colitis is provoked by the locally activated lymphocytes. Literature show that the numbers of epithelial cells, goblet cells, and glands will decrease compared with controls (Hibi *et al.,* 2002).

3.2.6 Assessment of Ulcerative Colitis

3.2.6.1 Body Weight, Stool Consistency and Occult Blood Assessment

Body weights of all rats were determined in two phases (**I** and **II)** using a suitable top-pan balance. Stool consistency and occult blood were also observed of all rats in same phases and scored arbitrarily as in Table 3.1 and Table 3.2 below.

Table 3.1 Scoring scale for stool consistency (Hagar *et al.*, 2007).

Assessment	Description	Score
Stool consistency	Well formed pellets	0
	Pasty and semi-formed stools	1
	Liquid stools	2

Table 3.2 Scoring scale for faecal occult blood (Hagar *et al.*, 2007).

Assessment	Description	Score
Faecal occult blood	No blood in haemoccult	0
	Positive haemoccult	1
	Gross bleeding	2

3.2.6.2 Biochemical Assessment

The entire colon of representative animals were retrieved from the anal region to colorectal junction, cut open longitudinally and rinsed in cold isotonic saline. Subsequently, 10 cm lengths were cut for biochemical assay while the remainders were fixed in Bouin's fluid for histopathological assessment.

The first 10 cm portion was subsequently weighed and the wet mass recorded in grams. It was then homogenized (1:10, w/v) in ice bath Phosphate buffer using polytron homogenizer (50 mg tissue/ml) and kept at -80°C until used for enzyme activity determination (Damiani *et al.*,2007). The homogenates were used to measure myeloperoxidase (MPO) activity and lipid peroxidation (Hagar *et al.*, 2007).

3.2.6.2.1 Colonic myeloperoxidase (MPO) activity

MPO activities were assessed as markers of neutrophil infiltration with the addition of 0.5% (w/v) cetrimide to colonic tissues homogenates contained in 10 mM sodium phosphate buffer (pH 7.4) and centrifuged for 30 min at 20,000 g/4 °C. Aliquots (1 ml) of the supernatants were then diluted in 1:10 of distilled water, to which 100 microliter collected in triplicates per sample. Subsequent 50 microliter of phosphate buffer was added to each well and a 25 micro-liter reaction mixture of; 95.2 mg O-dianisidine in 30 ml of water and 84 ml of 30% H_2O_2 in 50 ml of water was finally introduced. The rate of change in absorbances was then measured spectrophotometrically at 450 nm (Hagar *et al.*, 2007).

3.2.6.2.2 Colonic Malondialdehyde (MDA) Titre

Lipid peroxidation, an indicator of mucosal injury induced by reactive oxygen species was measured as thiobarbituric acid reactive substance (TBARS) by reacting 0.5 ml of colonic tissue homogenates with 1 ml of 10% trichloroacetic acid (TCA), 1 ml of 0.67% thiobarbituric acid (TBA) and 0.25 M HCl. Samples were subsequently boiled for 15 min, cooled and centrifuged. To these, 100 microliter triplicates were collected per sample, with subsequent additions of 50 microliter of phosphate buffer and the absorbance of the supernatants were then spectrophotometrically measured at 450 nm (Hagar *et al.*, 2007).

3.2.6.3 Histological Assessment

For histological examinations, excised colon of the rats were slightly cleaned in physiological saline, fixed in Bouin's fluid, dehydrated in increasing concentrations of ethanol and embedded in paraffin wax. Thereafter, sections of tissue were cut at 5 μm with a rotary microtome, mounted on clean glass slides and stained with haematoxylin and eosin for evaluation at magnifications X 100 (Chung, 2007).

3.2.6.4 Macroscopic and Microscopic Scoring

Colonic mucosal damage index was quantified by the clinical scoring system and assigned values based on an arbitrary scale as in Table 2.3

Table 3.3 Scoring scale for colonic damage (Hagar *et al.*, 2007).

Assessment	Description	Score
Colonic Damage	No macroscopic changes	0
	Mucosal erythema only	1
	Mild mucosal oedema, slight bleeding or small erosions	2
	Moderate oedema, slight bleeding ulcers or erosions	3
	Severe ulceration, oedema and tissue necrosis	4

3.2.6.5 Statistical Methods

All data collected were subjected to Graph-Pad Prism (software package), analysing differences between groups using Bonferroni's post-tests of two-way ANOVA or Newman-Keuls test of one-way ANOVA where applicable. The mean ± S.E.M of each animal group was then compared against the normal and disease controls, with possibility values of

3.3 RESULTS

3.3.1 Effect of TMBE on colonic macroscopic scores

The ethanolic extract on indomethacin-induced ulcerative colitis ($TMBE_{Et}$ on indo-induced UC), revealed colonic ulceration in the excised colon segments as in Fig 3.8, where the naive animal group showed absence of ulcers, the disease control group revealed distorted and swollen mucosa. Remission was dose dependent in the $TMBE_{Et}$ treated groups. There was significant remission in all other groups ($P > 0.001$) relative to the naives and remission of ulcers was even better in the standard control and 300 mg/kg group ($P > 0.01$). Ulcers of the $TMBE_{Et}$ treated groups had also significantly regressed ($P < 0.001$) relative to the diseased untreated (See Fig 3.6a).

In the ethanolic extract on acetic acid induced ulcerative colitis ($TMBE_{Et}$ on AA-induced UC), ulceration was clearly manifested in Fig 3.8 – 3.10, with micrographs of the colons for the normal control animals showing highly convoluted mucosa with no evidence of ulceration and those of the disease control, mildly eroded with swollen mucosae (Fig 3.8). Data for $TMBE_{Et}$ on AA-induced UC showed that remission of ulcer was significant ($P < 0.001$) among the 30 mg/kg and 100 mg/kg groups against the normal control. Similarly ulcers of the 30 mg/kg $TMBE_{Et}$ had significantly regressed ($P > 0.01$), those of the standard control, 100 mg/kg and 300 mg/kg $TMBE_{Et}$ groups had all significantly regressed ($P < 0.001$) relative to the diseased control (Fig 3.6b).

The study of aqueous extract on acetic acid induced ulcerative colitis ($TMBE_{Aq}$ on AA-induced UC) showed remission of mucosal ulceration as in Fig 3.6c & 3.7c, pointing to a dose dependent response.

Macroscopic scores of the colons showed severe ulceration ($P > 0.001$) in the disease untreated and 30 mg/kg, while ulcers of the 100 mg/kg and 300 mg/kg $TMBE_{Aq}$ had significant remitted ($P > 0.05$). The degree of remission had appreciated ($P > 0.01$) among the standard treated relative to the naive group. Compared to the diseased control, ulcers of the standard treatment had significantly remitted ($P < 0.001$) the degree of remission dropped marginally ($P < 0.01$) for the 30 mg/kg $TMBE_{Aq}$ treated group but was highly ($P > 0.001$) for the 100 mg/kg and 300 mg/kg (See Fig 3.6c).

As seen in Fig 3.6d, evaluation of colonic damage showed increased score in the disease control group for the petroleum ether extract on acetic acid induced ulcerative colitis ($TMBE_{Pt}$ on AA-induced UC). There was a decrease in colonic damage scores for the standard (Prednisolone) and *Trichilia* treated groups but which was not sustained over the experimental period. Meanwhile, the macroscopic scores of all other groups against the naive animals were found to have significantly reduced for the diseased, 100 mg/kg and 300 mg/kg $TMBE_{Pt}$ ($P < 0.001$). But the degree of ulcer regression dropped ($P < 0.01$) for the standard and 30 mg/kg ($P < 0.05$) treatments. Remission of ulcer in the standard and 300 mg/kg treated groups had however significantly appreciated ($P < 0.001$) relative to the disease control, but not for the 30 mg/kg and 100 mg/kg treatments. Colonic damage persisted through to the 300 mg/kg dose *Trichilia* treated group as shown in Fig 3.10g. Colonic damage of the ethyl acetate extract on acetic acid induced ulcerative colitis ($TMBE_{Ea}$ on AA-induced UC) showed higher scores in the *Trichilia* treated group as with the disease control rats. There was a decrease in colonic damage score for the prednisolone treated group (See Fig 3.6e). Colonic scores of $TMBE_{Ea}$ on AA-induced UC revealed some significant increment for all the animal groups ($P < 0.001$) but the magnitude fell for standard treated group ($P < 0.01$) against the naive animal group.

On the other hand, the degree of remission in the standard and 100 mg/kg treatment groups had significantly differed from the diseased ($P < 0.001$), decreasing at $P < 0.01$ in the 30 mg/kg but with no significant remission in the 300 mg/kg relative to the diseased group ($P > 0.05$). Colonic damage notably increased with increasing dose among the *Trichilia* treated groups as shown in Fig 3.6e.

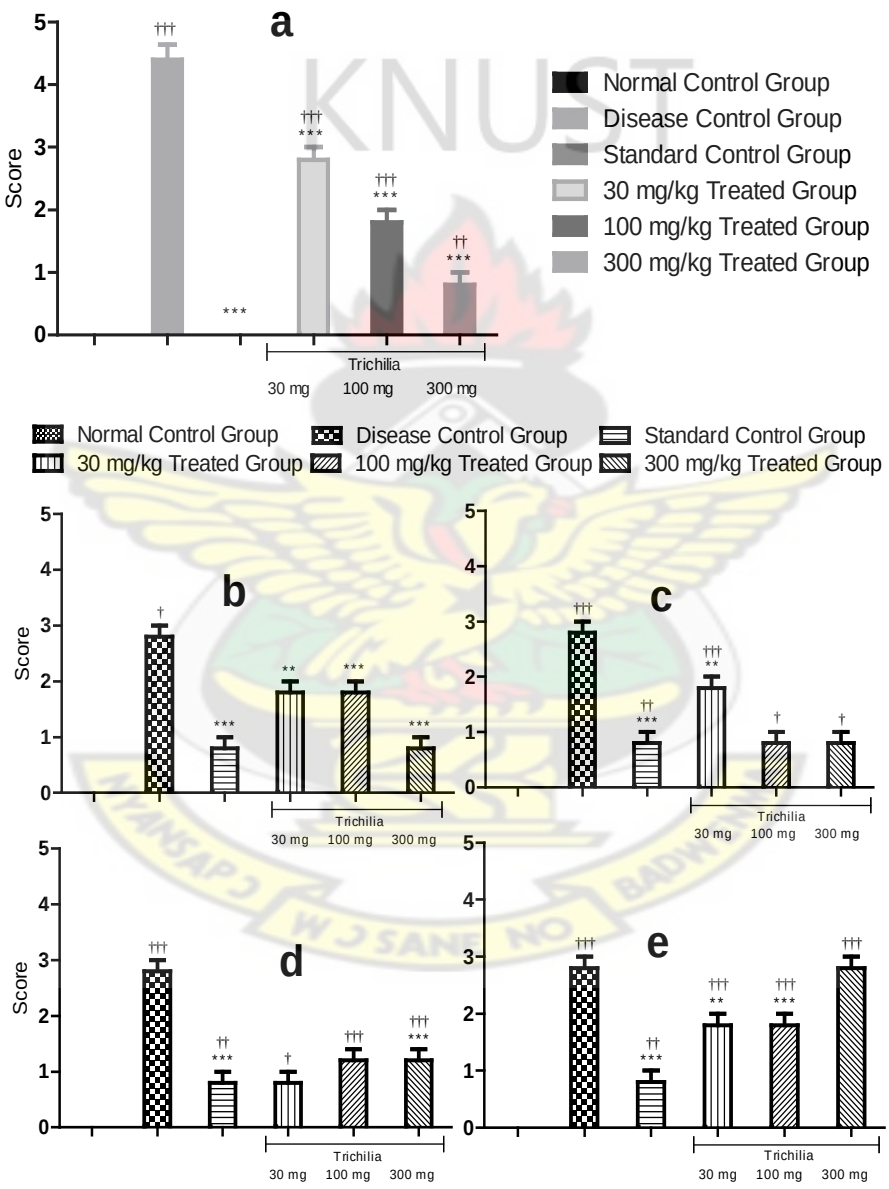


Figure 3.6: Effect of TMBE on Colonic macroscopic scores. **(a)** $TMBE_{Et}$ on indo-induced UC, **(b)** $TMBE_{Et}$ on AA-induced UC, **(c)** $TMBE_{Aq}$ on AA-induced UC, **(d)** $TMBE_{Pt}$ on AA-induced UC, **(e)** $TMBE_{Ea}$ on AA-induced

UC. **Note:** (n=5, using Newman-Keuls test and comparing the mean ± S.E.M of each group against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the disease control)

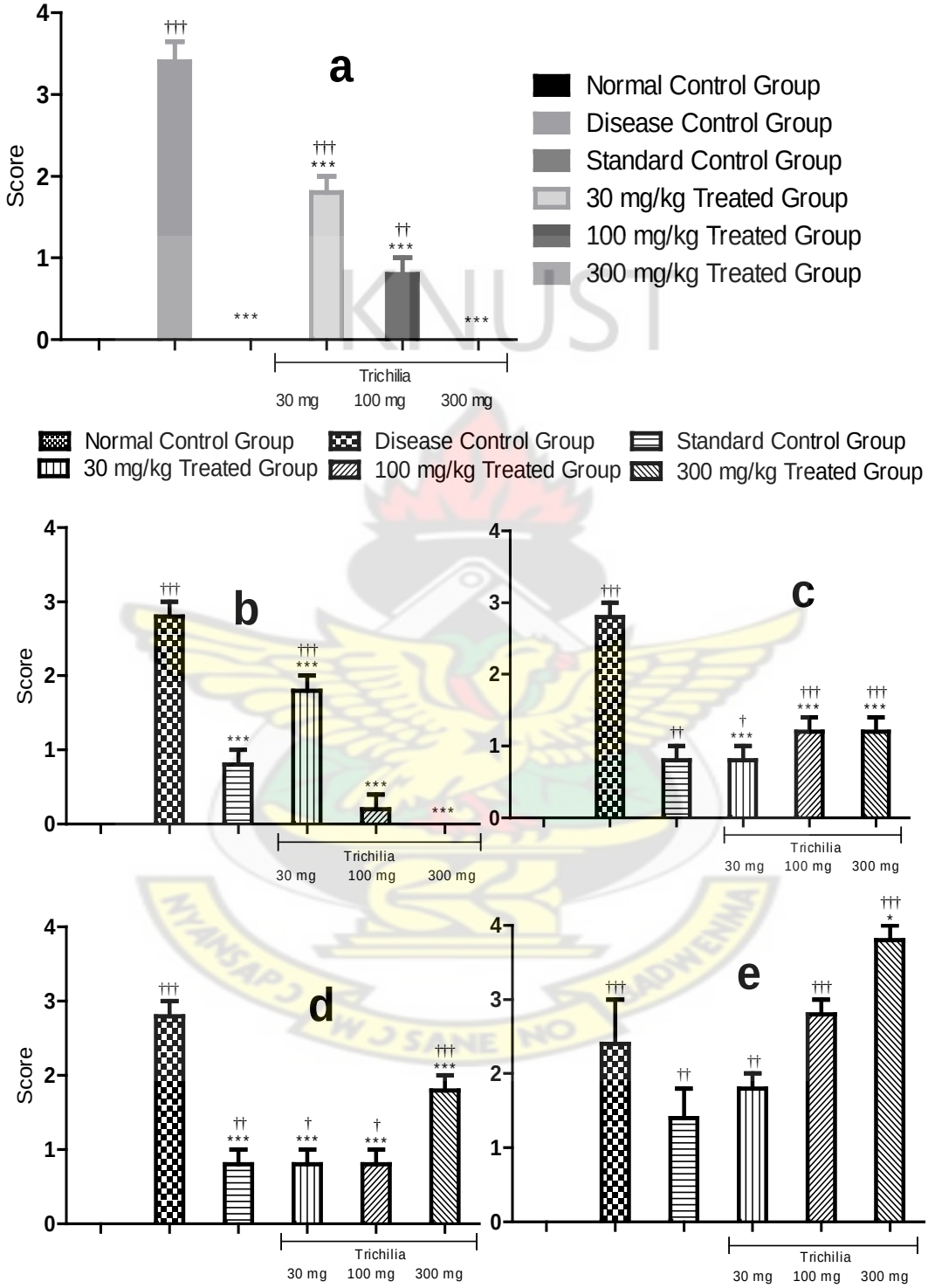


Figure 3.7: Effect of TMBE on Colonic microscopic scores. **(a)** $TMBE_{Et}$ on indo-induced UC, **(b)** $TMBE_{Et}$ on AA-induced UC, **(c)** $TMBE_{Aq}$ on AA-induced UC, **(d)** $TMBE_{Pt}$ on AA-induced UC, **(e)** $TMBE_{Ea}$on AA-induced

UC. **Note:** (Each column represents the mean ± S.E.M. n=5, using Newman-Keuls test and comparing the means of each animal group against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the disease control)

3.3.2 Effect of TMBE on colonic microscopic scores

Colonic ulceration regressed in the $TMBE_{Et}$ on indo-induced UC in a dose dependent fashion. There were few ulcers and epithelialisation of mucosa in the 30 mg/kg treated group (Fig 3.8). Epithelialisation and mucosal convolution were also observed in the 100 mg/kg treated group (Fig 3.9) and the endothelia of the 300 mg/kg dose treated group had been restored with no ulceration (Fig 3.10). Colonic microscopic scores were persistently high for the diseased and 30 mg/kg treated groups ($P < 0.001$) but had significantly reduced for the 100 mg/kg ($P < 0.01$) and was insignificant for the standard and 300 mg/kg treated groups ($P > 0.05$) relative to the naive animal group. Ulceration in the $TMBE_{Et}$ treated groups had also significantly ($P < 0.001$) regressed relative to the diseased untreated (see Fig 3.7a).

Colonic micrographs of the $TMBE_{Et}$ on AA-induced UC manifested a dose dependent healing, consistent with data for wet colonic weights in such a way that the 30 mg/kg $TMBE_{Et}$ showed mildly eroded and slightly oedematous mucosa (Fig 3.8). The 100 mg/kg treated group showed marked epithelialisation and mucosal convolution (Fig 3.9) while the 300 mg/kg $TMBE_{Et}$ group had a restored and highly convoluted mucosa, a manifestation of complete healing (Fig 3.10). The microscopic scores for the diseased and 30 mg/kg $TMBE_{Et}$ groups were found to have significantly increased ($P < 0.001$) compared to the naives, but not for the 100 mg/kg and 300 mg/kg groups ($P > 0.05$). Interestingly, ulcers of the $TMBE_{Et}$ treated groups had all significantly remitted relative to the diseased untreated ($P < 0.001$) as shown in Fig 3.7b. The standard treated group however, had erythematous mucosae with intact submucosa and muscularis (see Fig 3.8).

Micrographs of colons for the $TMBE_{Aq}$ on AA-induced UC showed anti-colitis effect with increasing dose. The 30 mg/kg treated group manifesting ulceration and oedema while the 100 mg/kg treated group showed mucosal columnisation, evident of pronounced recovery and the 300 mg/kg dose treated group, restored mucosal integrity. On the contrary, the disease control group had highly serrated mucosae, depicting severe ulceration relative to the normal control group which had convoluted mucosae with no evidence of ulceration as shown in Fig 3.10. The microscopic scores of ulceration were significant for the diseased, 100 mg/kg and 300 mg/kg $TMBE_{Aq}$ treated groups ($P < 0.001$), dropping in the 30 mg/kg dose and the standard treated groups ($P < 0.05$ and $P < 0.01$). Interestingly, ulcers of all the $TMBE_{Aq}$ and prednisolone treated groups had significantly healed ($P < 0.001$) relative to the diseased group (see Fig 3.7c)

The micrographs as in Fig 3.10g, showed highly cryptic and globular mucosae for the 300 mg/kg dose treated group, remitting with decreasing dose. The 100 mg/kg treated group had reduced cryptic abscesses (Fig 3.9g) and the 30 mg/kg treated group had intact mucosae with just few crypt abscesses (Fig 3.8g). Mucosae of the standard treated group were globular and oedematous while mucosae of the disease control group were highly serrated with crypt abscesses. The normal control group on the other hand had convoluted mucosae with no evidence of ulceration as in Fig 3.8 - 3.10. Microscopic scores of $TMBE_{Pt}$ on AA-induced UC were significantly high for the diseased and 300 mg/kg treatment ($P < 0.001$), but the degree of ulceration decreased ($P < 0.01$) in the standard control, and in the 30 mg/kg and 100 mg/kg groups ($P < 0.05$) relative to the naives. Ulceration in all treatment groups were however significantly reduced relative to the diseased ($P < 0.001$) as shown in Fig 3.7d.

The micrographs in Fig 3.8h, illustrates highly serrated and cryptic mucosae in the disease control group, globular and oedematous mucosae in the standard treated group, ulcerated and distorted mucosae in the 30 mg/kg treated group (Fig 3.8h). While the mucosae of the 100 mg/kg treated group were filled with crypt abscesses and oedema (Fig 3.9h), the 300 mg/kg dose treated group had serrated mucosae (Fig 3.10h). Microscopic colonic scores of $TMBE_{Ea}$ on AA-induced UC revealed persistent ulceration for all the animal groups ($P < 0.001$), remitting slightly in the standard treated ($P < 0.01$) against the naive animals, and only regressed marginally for the 300 mg/kg against the diseased ($P < 0.05$) as in Fig 3.7e.

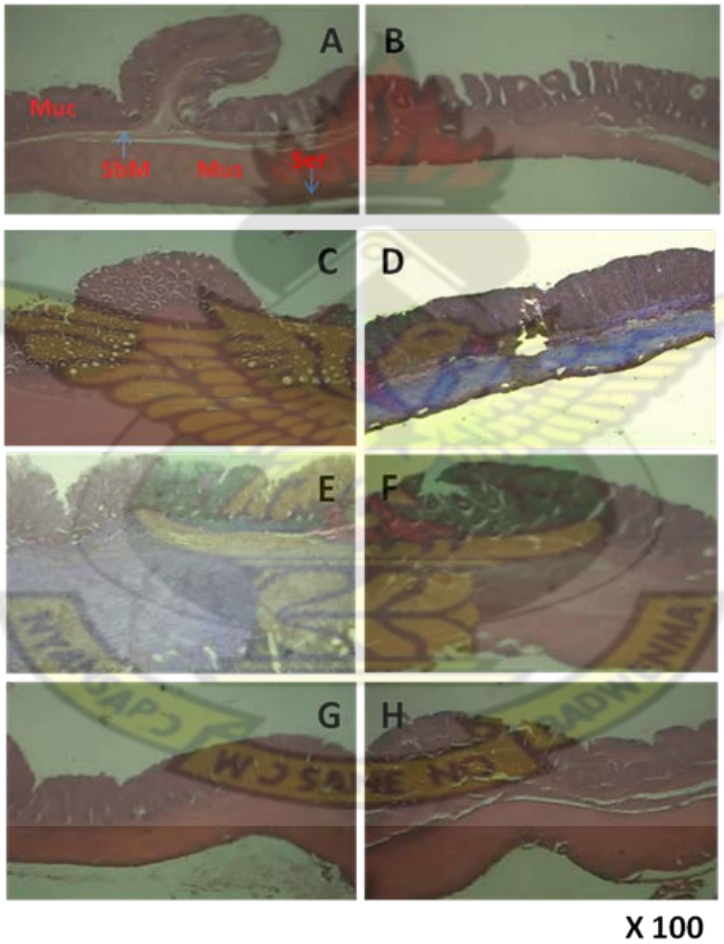


Figure 3.8: The Effect of 30 mg/kg TMBE on Colonic Ulcers. (A) = Normal Control Group; Convoluted Mucosa with No Evidence of Ulceration, (B) = Disease Control Group; Highly Serrated & Cryptic Mucosa, Evident of Severe Ulceration, (C) = Standard Control Group; Globular & Oedematous Mucosa, Evident of Recovery, (D) = $TMBE_{Et}$ on indo-induced UC; Epithelialisation of Mucosa, Evident of healing (E) = $TMBE_{Et}$ on AA-induce UC; Mildly eroded & Slightly Oedematous Mucosa, Evident of Slow Healing (F) = $TMBE_{Aq}$ on AA-induce UC; Ulceration with Oedema, Evident of Slow Healing (G) = $TMBE_{Pt}$ on AA-induce UC; Restored Mucosa with few Crypts, Evident of Gradual Healing (H) = $TMBE_{Ea}$ on AA-induce UC; Ulcerated & Distorted Mucosa, Evident of Disease. **Note:** Muc; Mucosa, SbM; Submucosa, Mus; Muscularis, Ser; Serosa.

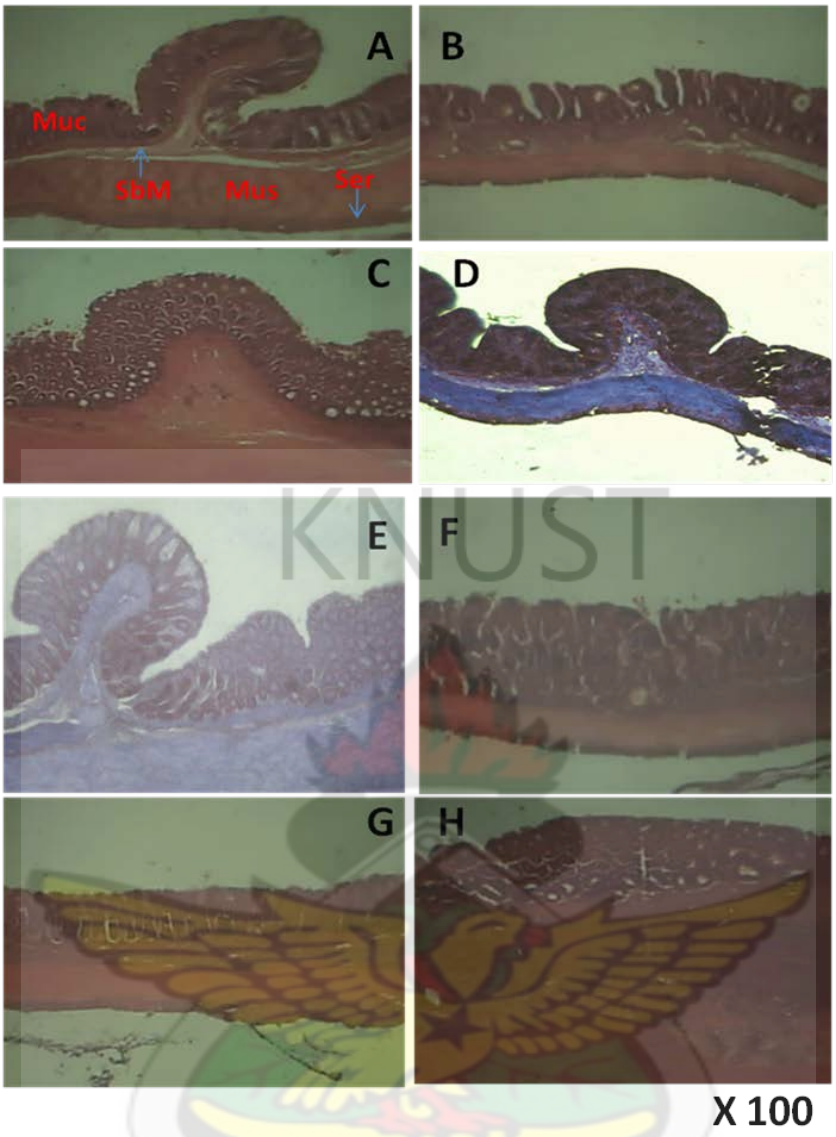


Figure 3.9: The Effect of 100 mg/kg TMBE on Colonic Ulcers; (A) = Normal Control Group; Convoluted Mucosa with No Evidence of Ulceration, (B) = Disease Control Group; Highly Serrated & Cryptic Mucosa, Evident of Severe Ulceration, (C) = Standard Control Group; Globular & Oedematous Mucosa, Evident of Recovery, (D) = $TMBE_{Et}$ on indo-induced UC; Epithelialisation & Mucosal Convolution, Evident of Pronounced Healing (E) = $TMBE_{Et}$ on AA-induce UC; Epithelialisation & Mucosal Convolution, Evident of Pronounced Healing (F) = $TMBE_{Aq}$ on AA-induce UC; Columnisation of Mucosal, Evident of Pronounced Recovery (G) = $TMBE_{Pt}$ on AA-induce UC; Cryptic Mucosa, Evident of Suspected Toxicity (H) = $TMBE_{Ea}$ on AA-induce UC; Cryptic Oedematous Mucosa, Evident of Inflammation. **Note:** Muc; Mucosa, SbM; Submucosa, Mus; Muscularis, Ser; Serosa.

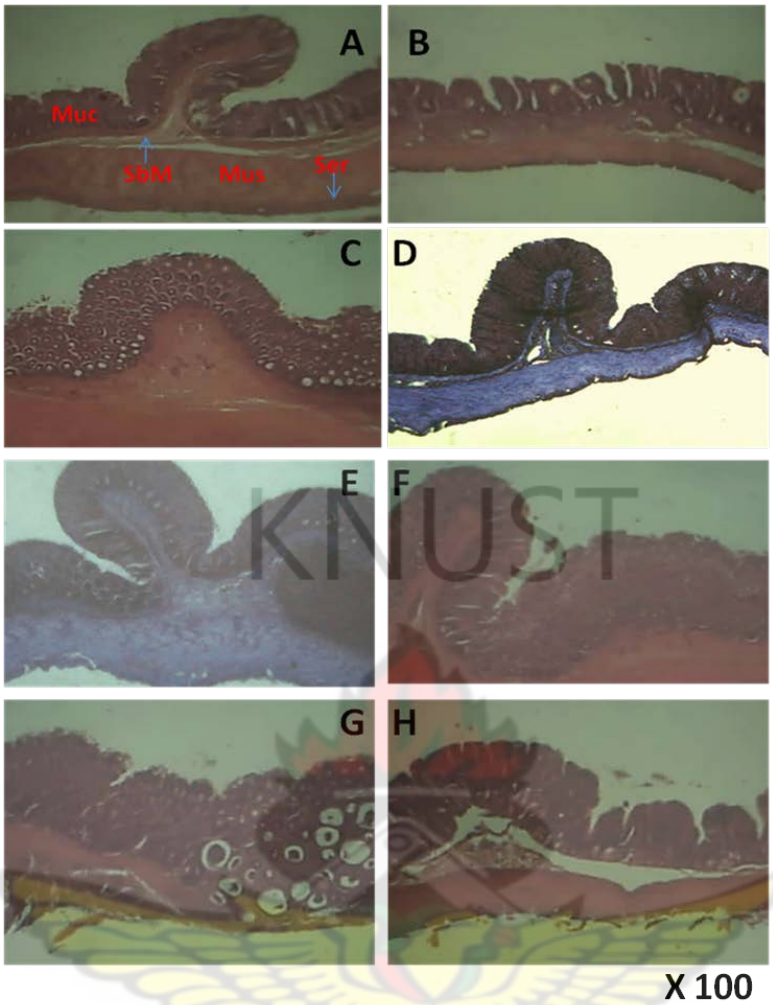


Figure 3.10: The Effect of 300 mg/kg TMBE on Colonic Ulcers; (A) = Normal Control Group; Convoluted Mucosa with No Evidence of Ulceration, (B) = Disease Control Group; Highly Serrated & Cryptic Mucosa, Evident of Severe Ulceration, (C) = Standard Control Group; Globular & Oedematous Mucosa, Evident of Recovery, (D) = $TMBE_{Et}$ on indo-induced UC; Restored endothelia; Mucosa & Submucosa Evident of Complete Healing (E) = $TMBE_{Et}$ on AA-induce UC; Restored & Highly Convoluted Mucosa, Evident of Complete Healing (F) = $TMBE_{Aq}$ on AA-induce UC; Restored Mucosal integrity, Evident of Pronounced Healing (G) = $TMBE_{Pt}$ on AA-induce UC; Highly Cryptic & Globular Mucosa, Evident of Highly Suspected Toxicity (H) = $TMBE_{Ea}$ on AA-induce UC; Serrated Mucosa, Evident of Poor Healing. **Note:** Muc; Mucosa, SbM; Submucosa, Mus; Muscularis, Ser; Serosa.

3.3.3 Effect of TMBE on Myeloperoxidase (MPO) and Malondialdehyde (MDA)

$TMBE_{Et}$ activity showed in both myeloperoxidase and malondialdehyde levels manifesting some antagonism in the 100 mg/kg treated groups but with appreciable activity in the 30 mg/k and 300 mg/kg dose *Trichilia* treated groups as in Fig 3.11a and Fig 3.12 a. There was no significant difference for the MPO activity in all animal groups against the normal of $TMBE_{Et}$ on AA-induced UC. But this was not the case with MDA titre (see Fig 3.11a & 3.12a).

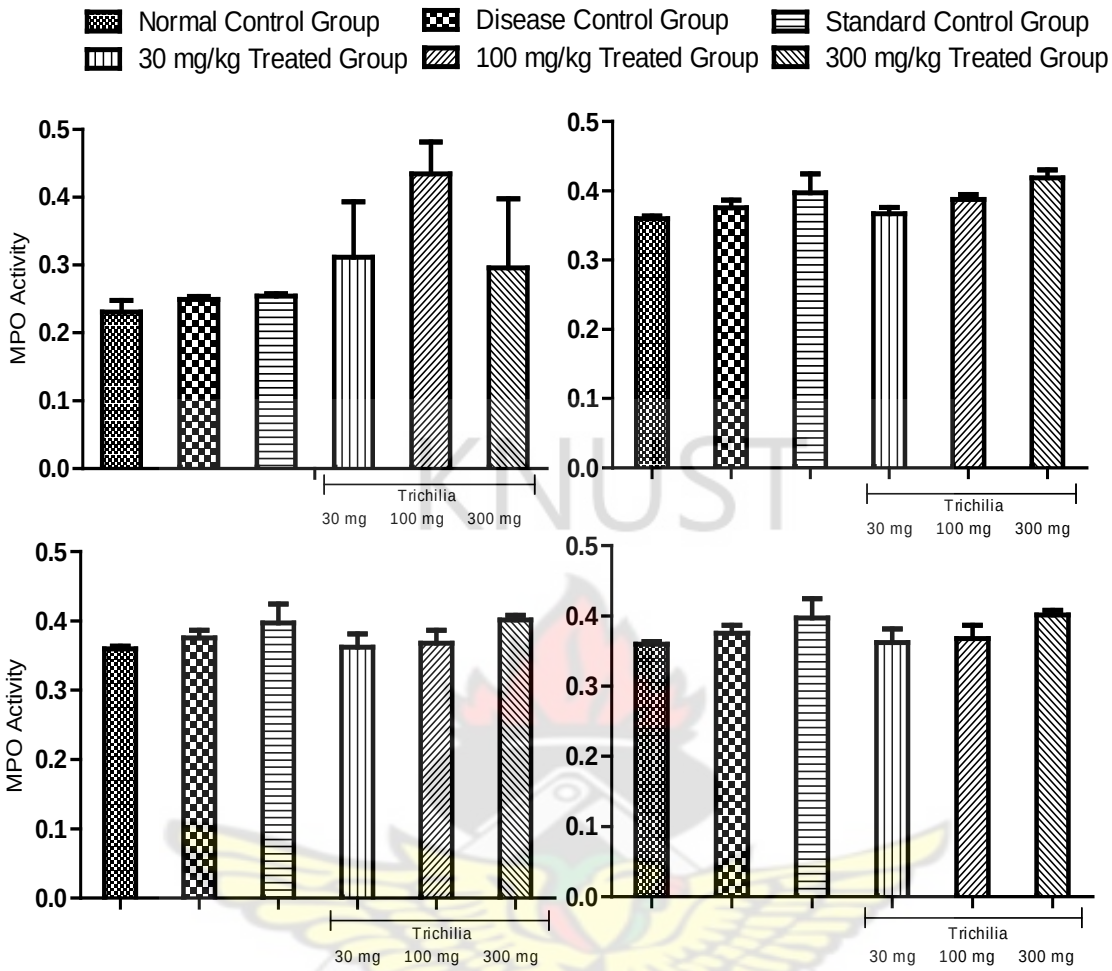


Figure 3.11: Effect of TMBE on Colonic myeloperoxidase activity. **(a)** $TMBE_{Et}$ on AA-induced UC, **(b)** $TMBE_{Aq}$ on AA-induced UC, **(c)** $TMBE_{Pt}$ on AA-induced UC, **(d)** $TMBE_{Ea}$ on AA-induced UC. **Note:** (n=5, using Newman-Keuls test and comparing the mean ± S.E.M of each animal group against the normal control, †††P < 0.001; ††P < 0.01, †P < 0.05, and ***P < 0.001; **P < 0.01, *P < 0.05 against the disease control)

The *Trichilia* bark extracts curtailed colonic inflammation as shown by the lower levels of malondialdehyde titres among the 30 mg/k, 100 mg/kg and 300 mg/kg TMBE treated groups (Fig 3.12) relative to the disease untreated group. MPO activity varied insignificantly in all other groups (P > 0.05) relative to the normal group (P > 0.05).

The situation was very different for MDA titres which showed significant differences of P < 0.01, for the 30 mg/kg and 300 mg/kg (Fig 3.12c & 3.12d) and P < 0.001 for 300 mg/kg of $TMBE_{Aq}$ as in Fig 3.12b. For the 100 mg/kg TMBE treated groups of $TMBE_{Et}$, $TMBE_{Pt}$, and $TMBE_{Ea}$ fractions, MDA levels dropped significantly at P < 0.001 as shown in Fig 3.12b, 3.12c & 3.12d. MDA titre decreased among animals of the standard treatment at P < 0.1 for all extracts, but for $TMBE_{Et}$ (Fig 3.12a) which showed a P < value of 0.05.

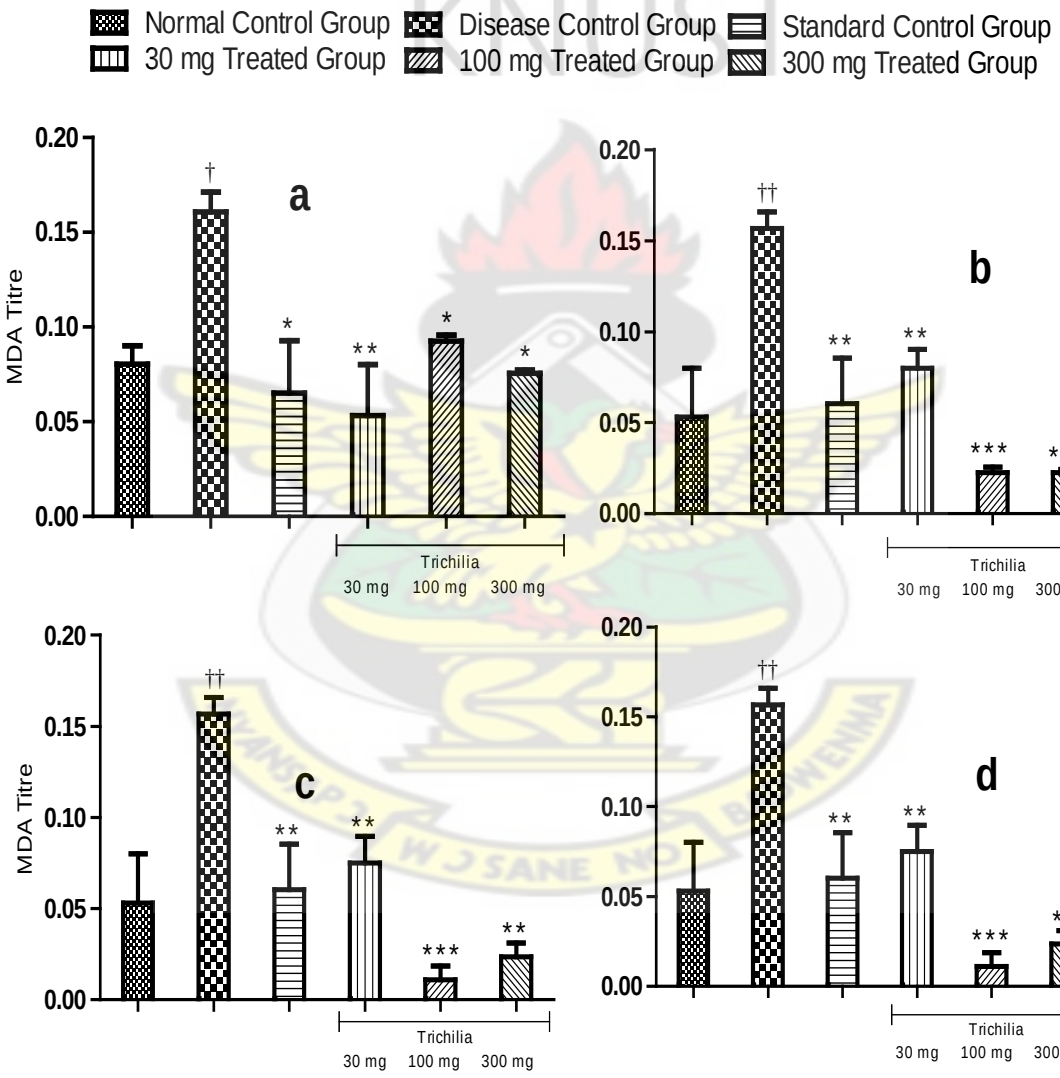


Figure 3.12: Effect of TMBE on Colonic malondialdehyde titre. **(a)** $TMBE_{Et}$ on AA-induced UC, **(b)** $TMBE_{Aq}$ on AA-induced UC, **(c)** $TMBE_{Pt}$ on AA-induced UC, (**d)** $TMBE_{Ea}$ on AA-induced UC. **Note:** (n=5, using Newman-Keuls test and comparing the mean ± S.E.M of each animal group against the normal control, †††P < 0.001; ††P < 0.01, †P < 0.05, and ***P < 0.001; **P < 0.01, *P < 0.05 against the disease control).

3.6.4 Effect of TMBE on wet colonic weight

Colon segments of all colitis induced groups of $TMBE_{Et}$ on indo-induced UC weighed heavier than normal colons of the normal group (Fig 3.13a). Wet weight of colons at equal lengths of 10 cm, tested with one-way ANOVA using the Newman-Keuls' multiple comparison test, showed that there were insignificant increases in colonic weight among all the animal groups ($P > 0.05$) against the normal/naive group. The trend was again dose dependent. Colonic segments of all colitis induced groups of $TMBE_{Et}$ on AA-induced UC were also heavier than normal colons of the naive group (Fig 3.13b). Treatment was effective in a dose dependent fashion among the *Trichilia* treated groups and most effective in the standard and 300 mg/kg treated group. Fig 3.13b however shows an expected much heavier colons among ($P < 0.05$) the disease control group, but the standard, 30 mg/kg, 100 mg/kg and 300 mg/kg $TMBE_{Et}$ which all increased insignificantly ($P > 0.05$) relative to the normal control group. Meanwhile, colons of the standard and 300 mg/kg $TMBE_{Et}$ had significant gained weight ($P < 0.01$) relative to the diseased, and while colonic weights of the 100 mg/kg had significantly increased ($P < 0.05$), those of the 30 mg/kg had not significantly differed from the diseased untreated (Fig 3.13b).

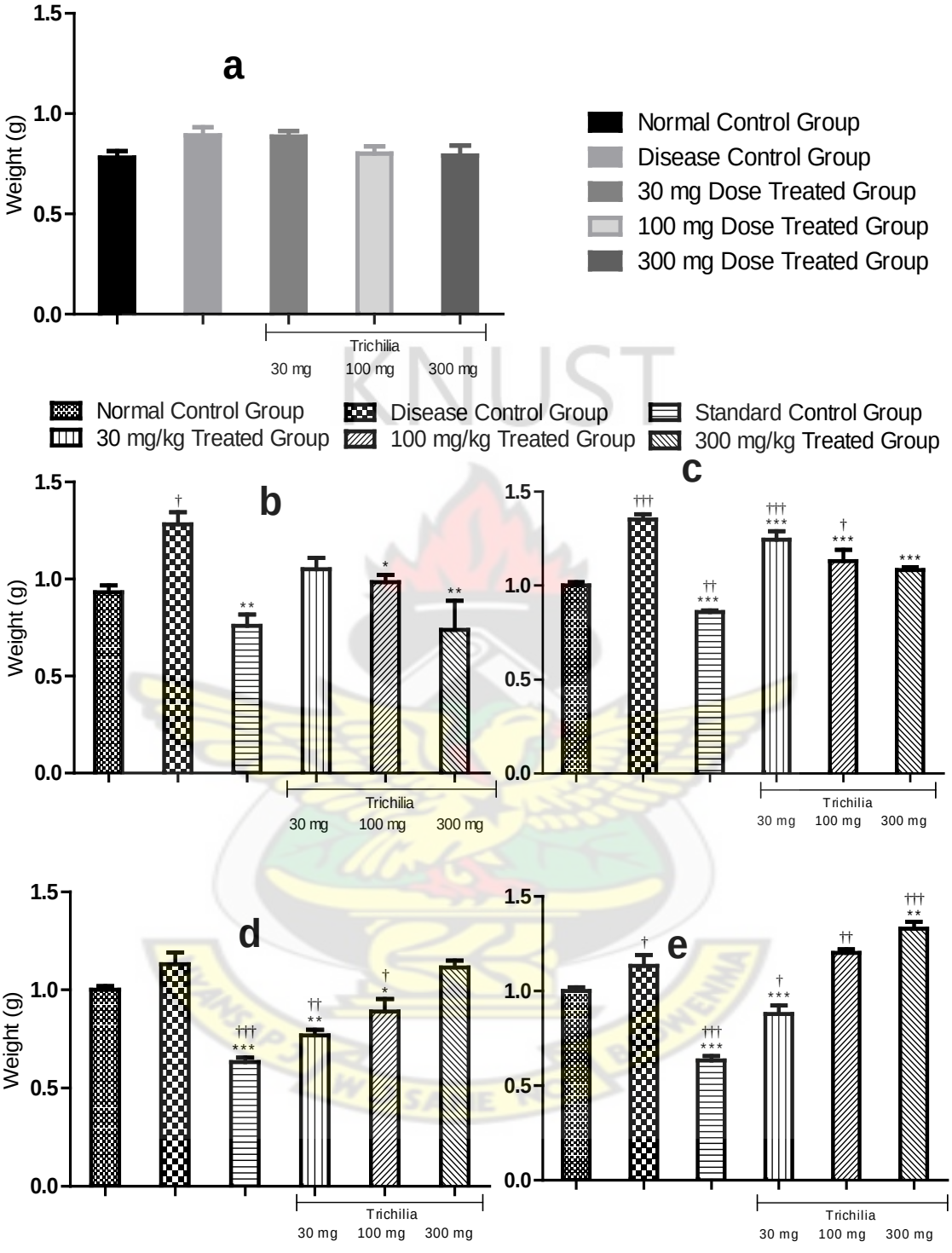


Figure 3.13: Effect of TMBE on Wet colonic weight. **(a)** $TMBE_{Et}$ on indo-induced UC, **(b)** $TMBE_{Et}$ on AA-induced UC, **(c)** $TMBE_{Aq}$ on AA-induce UC, **(d)** $TMBE_{P}$ on AA-induce UC, **(e)** $TMBE_{Ea}$ on AA-induce UC. **Note:** (n=5, using Newman-Keuls test and comparing the mean ± S.E.M of each animal group against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the disease control)

Colonic weight of $TMBE_{Aq}$ on AA-induced UC, on the other hand were significantly reduced compared to controls that received the vehicle. Wet colonic weights reduced in a dose dependent fashion among the *Trichilia* treated groups and most effective in the standard treated group but not the disease control group as shown in Fig 3.13c. The Newman-Keuls' tests showed significant increases ($P < 0.001$) for the diseased and 30 mg/kg $TMBE_{Aq}$. But while colonic weights of the standard control and the 100 mg/kg were appreciating significantly ($P < 0.01$ and $P < 0.05$), the 300 mg/kg $TMBE_{Aq}$ group showed no significant increase ($P > 0.05$) in colonic weight relative to those of the normal/naive group. All the $TMBE_{Aq}$ groups as well as the standard (Prednisolone) treated had drastically reduced ($P < .001$) in colonic weights relative to the diseased (Fig 3.13c).

Wet colonic weights of $TMBE_{Pt}$ treated groups increased with increasing dose relative to the standard treated group which registered a marked reduction in colonic weight relative to the naive animals. The disease control group pointed to severe erythema and oedema as shown in Fig 3.10g. Comparison of each other group against the naives for $TMBE_{Pt}$ on AA-induced UC rightly revealed significant increases in colonic weights for the standard and 30 mg respectively ($P < 0.001$ and < 0.01). While the diseased and 300 mg/kg $TMBE_{Aq}$ all showed no significant increases ($P > 0.05$), colonic weights of the 100 mg/kg had increased significantly ($P < 0.05$). In much the same way, the standard, 30 mg/kg, 100 mg/kg and 300 mg/kg $TMBE_{Aq}$ had increments in their respective colonic weights ($P < 0.001$, $P < 0.01$, $P > 0.05$) relative to the disease control as shown in Fig 3.13d.

Colonic weights of $TMBE_{Ea}$ treated groups similarly, increased in a dose dependent fashion among the *Trichilia* treated groups relative to the standard treated group which registered a marked reduction in colonic weight comparable to the naive animals. The disease control group on the other hand was reasonably heavy due erythema and oedema as shown in Fig 3.13e. Each other animal group of ethyl acetate extract ($TMBE_{Ea}$) showed significant increments in the standard and 300 mg/kg $TMBE_{Ea}$ relative the naive group ($P > 0.001$). Colonic weights of the 100 mg/kg $TMBE_{Ea}$ also increased ($P > 0.01$), with those of the diseased and 30 mg/kg increasing likewise ($P > 0.05$) relative to those of the naives. In comparison with the diseased control, the standard and 30 mg/kg treatments had significant increases in their colonic weight ($P > 0.001$), same was the case in the 300 mg/kg group ($P > 0.01$) while those of the 100 mg/kg remained without any significant increment ($P > 0.05$) as shown in Fig 3.13c.

3.3.5 Effect of TMBE on faecal occult blood

Animal faecal occult blood of $TMBE_{Et}$ on indo-induced UC showed complimentary evidence of ulceration and bleeding in the disease control and 30 mg/kg $TMBE_{Et}$ animal groups. The trend in Fig 3.14a points to a dose dependent pharmacological effect. Both the disease control and 30 mg/kg treated groups ($P < 0.001$) bled consistently. The 100 mg/kg and 300 mg/kg dose treated groups however showed significant improvement ($P > 0.05$). Meanwhile, the $TMBE_{Et}$ treated groups had all significantly improved relative to the diseased untreated ($P < 0.001$) as evident in Fig 3.14a.

Faecal occult blood test of $TMBE_{Et}$ on AA-induced UC had improved ($P > 0.05$) in all animal groups but the disease control (disease untreated), which was significant bleeding relative to normal control group and to the $TMBE_{Et}$ treated groups ($P < 0.001$). This was visible in the low and 100 mg/kg $TMBE_{Et}$ animal groups of $TMBE_{Et}$ but improved relative to the disease control group whose faecal occult blood soared over the experimental period. The trend in Fig 3.14b points to a dose dependent effect with the 300 mg/kg dose *Trichilia* group responding similarly to the standard treated group.

Occult blood of $TMBE_{Aq}$ on AA-induced UC however deviated from the trend of drug activity (Fig 3.14c). This was however not the case in the naive animal group and the standard treated group. It pointed to significant bleeding for the disease control relative to the naive animals ($P < 0.001$). The standard, 30 mg/kg and 100 mg/kg dose $TMBE_{Aq}$ treated groups had significant reduction in bleeding instances relative to the diseased animal group ($P < 0.01$) and as well as the 300 mg/kg $TMBE_{Aq}$ treatment ($P < 0.001$) as against the diseased.

The disease control, 100 mg/kg and 300 mg/kg dose groups of $TMBE_{Pt}$ on AA-induced UC all had significant increases in faecal occult blood examination ($P < 0.001$). The 30 mg/kg and standard treated groups marginally reduced bleeding in varying degrees ($P < 0.01$ and > 0.05) relative to the naive animal group. Unlike the standard treatment which had significant reduction, all the $TMBE_{Pt}$ treated groups had insignificantly reduced relative to the disease control ($P > 0.05$) as shown in Fig 3.14d.

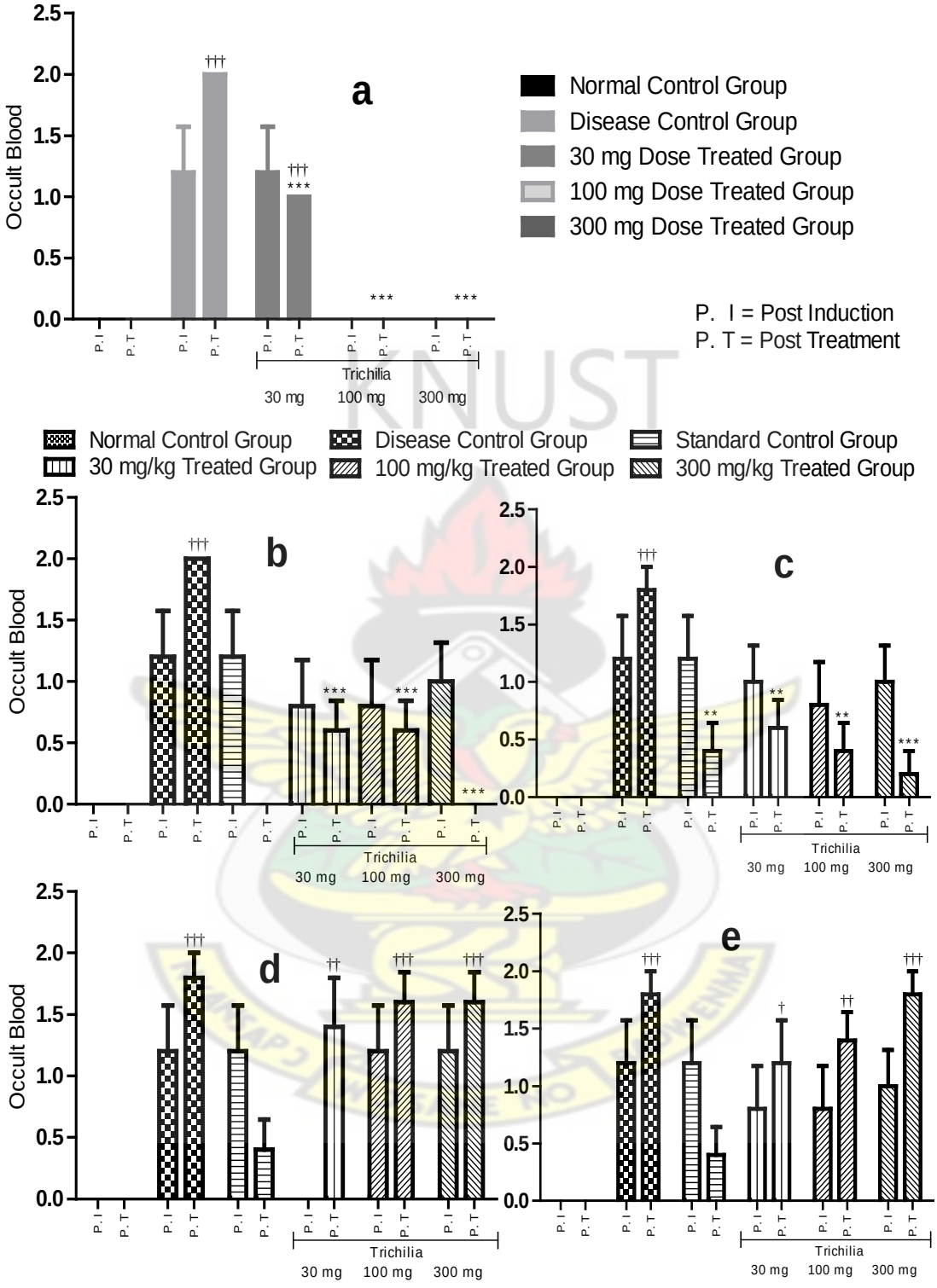


Figure 3.14: Effect of TMBE on Faecal occult blood. **(a)** $TMBE_{Et}$ on indo-induced UC, **(b)** $TMBE_{Et}$ on AA-induce UC, **(c)** $TMBE_{Aq}$ on AA-induce UC, **(d)** $TMBE_{Pt}$ on AA-induce UC, **(e)** $TMBE_{Ea}$ on AA-induce UC **Note:** (n=5, using Bonferroni's post-tests and comparing the mean ± S.E.M of each animal group's P.T against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the disease control)

3.3.6 Effect of TMBE on animal stool consistency

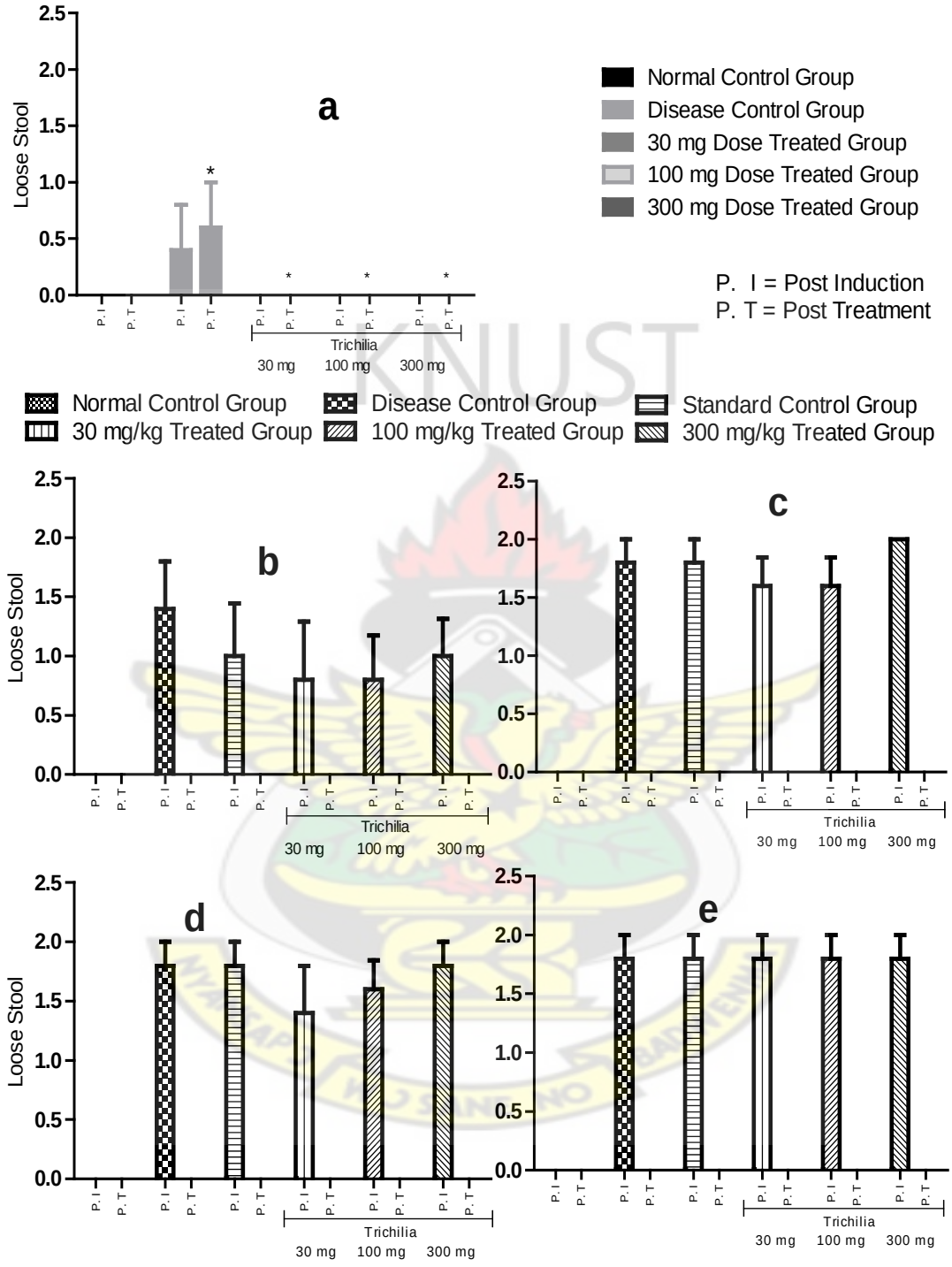


Figure 3.15: Effect of TMBE on Animal stool consistency. **(a)** $TMBE_{Et}$ on indo-induced UC, **(b)** $TMBE_{Et}$ on AA-induce UC, **(c)** $TMBE_{Aq}$ on AA-induce UC, **(d)** $TMBE_{Pt}$ on AA-induce UC, **(e)** $TMBE_{Ea}$ on AA-induce UC. **Note:** (n=5, using Bonferroni's post-tests and comparing the mean ± S.E.M of each animal group's P.T against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the disease control)

Faecal occult blood increased with increasing dose among the $TMBE_{Ea}$ treated groups. It was much severe in the disease control group similar to the 300 mg/kg dose treated group, but marginally decreased in the standard treated group as shown in Fig 3.14e. There however, were significant differences between the faecal occult blood of the disease control and 300 mg/kg dose treated groups as against the naive group ($P < 0.001$). Same could not be said for the standard treated, 30 mg/kg and 100 mg/kg treated groups ($P < 0.05$ and < 0.01). However, faecal occult blood of the standard treated had not significantly increased ($P > 0.05$. Meanwhile, all $TMBE_{Ea}$ groups bled as with the diseased control (See Fig 3.14e).

Evidence of recovery was obvious in the animal stool consistency examination as in Fig 3.15, which shows well-formed pellets for all *Trichilia* treated groups similar to stools of naive animals. The disease control group of $TMBE_{Et}$ on indo-induced UC (Fig 3.15a) had persistent diarrhoea all through the experimental period due to persistent pathology associated with untreated colitis. In comparison with the naives, diarrhoea persisted ($P < 0.05$) in the disease control group but not for the $TMBE_{Et}$ treated groups, which had significantly reduced relative to the diseased untreated.

Diarrhoea in the $TMBE_{Et}$ on AA-induced UC reduced as seen in Fig 3.15b, well-formed pellets were observed for all animal groups similar to stools of naive animals. Bonferroni's tests for $TMBE_{Et}$ on AA-induced UC showed no significant diarrhoea by the end of treatment ($P > 0.05$). Similarly, there were well-formed pellets for all animal groups of the $TMBE_{Aq}$ on AA-induced UC as in Fig 3.15c. There was no significant diarrhoea all the groups under consideration for $TMBE_{Aq}$ on AA-induced UC. Same was the case with $TMBE_{Pt}$ and $TMBE_{Ea}$ activities on AA-induced UC ($P > 0.05$).

Diarrhoea associated acetic-acid induced ulceration showed well-formed pellets for all animal groups of $TMBE_{Pt}$ on AA-induced UC post treatment as in Fig 3.15d, even though same could not be said post induction. There was diarrhoea in all animal groups post induction in the $TMBE_{Ea}$ on AA-induced UC but the naives. However, all the animal groups had demonstrated well-formed pellets for all animal groups post treatment as in Fig 3.15e.

3.3.7 Effect of TMBE on animal body weight

General body weights of experimental animals in the $TMBE_{Et}$ on indo-induced UC, dropped in all the groups but the naive animals, which showed a normal growth pattern as shown in Fig 3.16a. Using Bonferroni's post-tests and comparing the means of each animal group against the naive and diseased groups, there was significant loss in body weight ($P < 0.001$) for all other animal groups relative to the naives.

In the $TMBE_{Et}$ on AA-induced UC, animal body weights of the *Trichilia* treated groups were consistently similar to those of the standard treated group, showing very minimal reduction relative to the disease control group. The naive animals however showed a normal growth pattern with increased body weight as shown in Fig 3.16b. The trend pointed to significant losses in body weight for the diseased, 30 mg/kg and 100 mg/kg animal groups ($P < 0.001$). The standard and 300 mg/kg $TMBE_{Et}$ groups on the other hand reduced marginally ($P < 0.01$).

The *Trichilia* treated groups of the $TMBE_{Aq}$ on AA-induced UC showed animal body weights similar to those of the standard treated group, with minimal drop relative to the disease control group. While the naive animals showed a slight increase in body weight as shown in Fig 3.16c, the disease control group had a marginal drop ($P < 0.05$) in body weight.

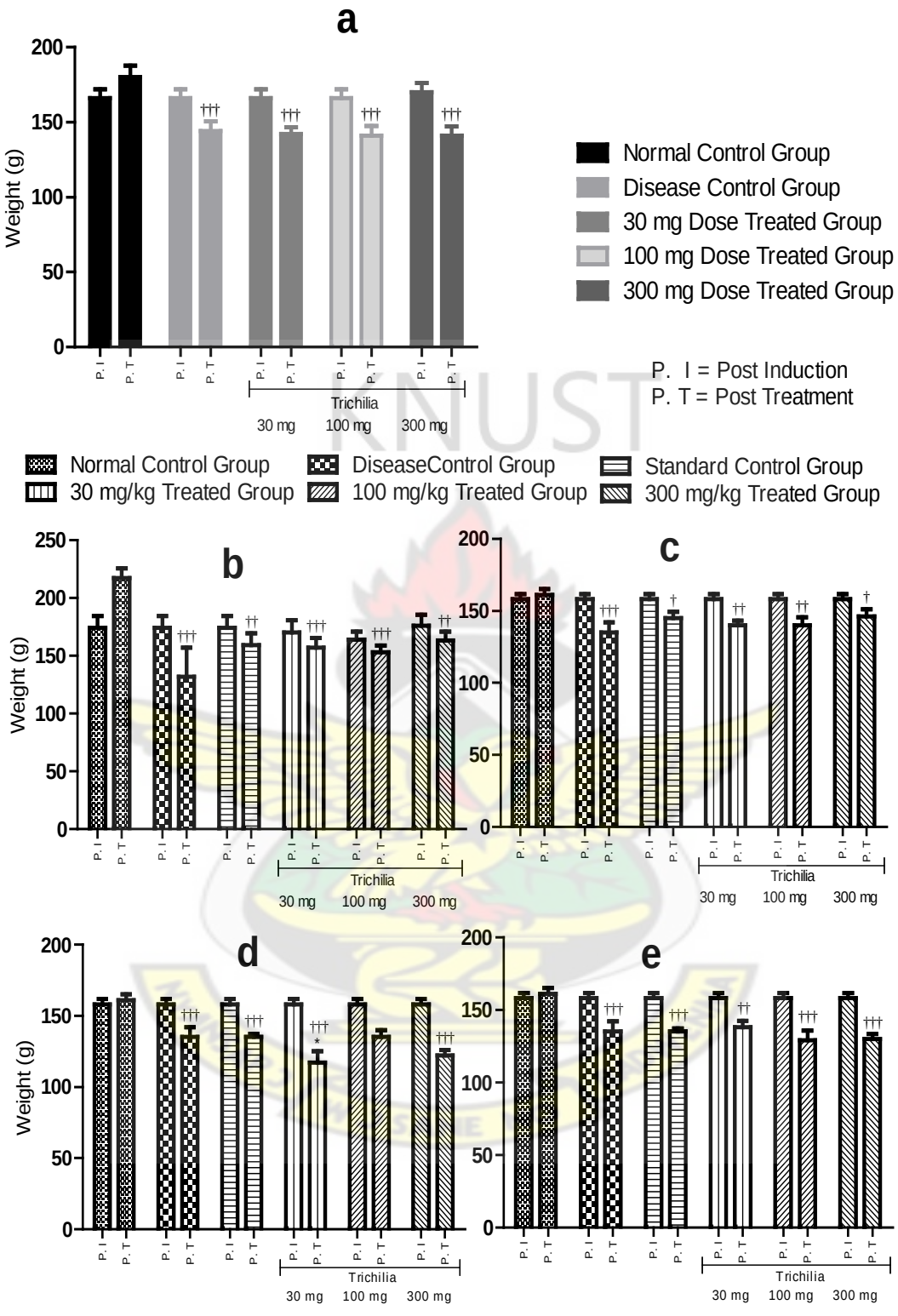


Figure 3.16: Effect of TMBE on Animal body weight.**(a)** $TMBE_{Et}$ on indo-induced UC, **(b)** $TMBE_{Et}$ on AA-induce UC, **(c)** $TMBE_{Aq}$ on AA-induce UC, **(d)** $TMBE_{Pt}$ on AA-induce UC, **(e)** $TMBE_{Ea}$ on AA-induce UC. **Note:** (n=5, using Bonferroni's post-tests and comparing the mean ± S.E.M of each animal group's P.T against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the disease control)

While there was significant losses ($P < 0.05$) in the body-weights of animals for the standard and 300 mg/kg dose groups relative to the normal. The diseased and 30 mg/kg $TMBE_{Aq}$ had significantly lost weights ($P < 0.001$) relative to the naives. In a dose dependent fashion, the 100 mg/kg had an appreciable reduction in body weights ($P < 0.01$) falling between the former groups. The change in body weight among the $TMBE_{Aq}$ groups had however not significantly differed from those of the disease control group as in Fig 3.16c.

Body weight reduced drastically among the *Trichilia* treated groups of the $TMBE_{Pt}$ on AA-induced UC in a severer manner relative to the disease control group. Only the naive animal group showed a slight increase in body weight as shown in Fig 3.16d. The disease control group had a marginal drop in body weight. There were significant losses ($P < 0.001$) in body weights of all animal groups against the naive animals, but for the 30 mg/kg treatment which had a slight reduction relative to the diseased ($P < 0.05$). All other treated groups had not significantly varied against the diseased ($P > 0.05$) except the 30 mg/kg treatment which showed significant reduction in body weight relative to the diseased ($P < 0.01$).

In the $TMBE_{Ea}$ on AA-induced UC, it was found that body weights stayed among the *Trichilia* treated groups in much the same way as in the disease control group. As shown in Fig 3.16e only the naive animals could sustain their body weights as from day 1 of experimentation. The disease control group had also dropped in body weight. Bonferroni also revealed significant body weight losses for all the other animal groups relative to the naive animal group of $TMBE_{Ea}$ on AA-induced UC.

3.4 DISCUSSION

Twenty-four hours after intracolonic administration of 3% acetic acid, the haematoxylin and eosin stained sections of colon showed an inflammatory response characterized by extensive destruction of mucosal epithelia due to principal free radical activity in the colonic tissues as suggested by Hagar *et al.*, (2006). Sustained production of reactive oxygen metabolites during colonic inflammation must have overwhelmed the endogenous antioxidant defence system that regulates their production leading to oxidative injury. In addition, endogenous antioxidants may be affected by acetic acid (Hagar *et al.*, 2006).

With respect to the indomethacin induced colitis, it is suggested that the depletion of endogenous prostaglandins (PGs) induced by indomethacin contributed to cause the typical ulcers; in fact, exogenous PGs prevent gastrointestinal damage in response to indomethacin. Enteritis induced by indomethacin occurs because both COX-1 and COX-2 are inhibited, leading to increased inducible NO synthase activity (Piepoli *et al.*, 2005). As such, in the $TMBE_{Et}$ on indo test, moderate to severe inflammation with large ulcers involving the entire colonic wall were observed. It is presumable that the source of oxidative stress in the disease could be traced to the activation of resident macrophages and in the subsequent afflux and activation of polymorphonuclear cells. Both these cells, once activated, are known producers of free radicals (Piepoli *et al.*, 2005).

Generally, diarrhoea and rectal bleeding occurred in day 1 and 2, following the induction of colitis. It has been recently been established that, the aqueous, alcoholic and petroleum ether extracts of the stem bark of *Trichilia monadelpha* have anti-inflammatory effects in animal models (Ainooson *et al.*,2012).

The administration of *Trichilia* bark extract was found to have ameliorated the pathogenesis of UC as shown in the aqueous and ethanolic extract treatments, consistent with the previous findings suggested by Ainooson *et al.*, (2012). The overall histological damage of colitis and TBARS activity were significantly reduced by *Trichilia* extract treatments. The wet colonic weights and colonic damage scores were reduced significantly in the aqueous and ethanolic extract treated group as compared to disease control group, relative to the normal control group. The colitis caused by acetic acid is associated with an increase in MPO activity and an enhanced level of MDA as indicated by Paiva *et al.*, (2005), which had decreased among animals of the *Trichilia* treatments relative to those of the disease untreated. In addition to the enhanced inflammatory infiltrate, extensive mucosal injury occurred, resulting in production and release of ROS, such as superoxide and hydrogen peroxide which is purported to have decreased among the *Trichilia* treated as marked by lower MDA levels.

These ROS interact in the presence of transition metals such as iron in order to generate the highly reactive and cytotoxic hydroxyl radical (Damiani *et al.*, 2007). Hydroxyl radical is capable of oxidizing and peroxidising a wide variety of biomolecules, such as proteins, carbohydrates, lipids and DNA. Activated neutrophils and monocytes also secrete the haemoprotein myeloperoxidase into the extracellular space, where it catalyzes the oxidation of chloride ions via hydrogen peroxide to yield the highly reactive oxidizing and chlorinating agent hypochlorous acid. The latter has been shown to degrade gastrointestinal mucin, enhance mucosal permeability, and injure intestinal epithelial cells (Damiani *et al.*, 2007), especially when endogenous anti-oxidant balance is lost. Therefore, the *Trichilia* bark extracts must have served as exogenous anti-oxidants making up for this ROS to anti-oxidant imbalance.

Pharmacotherapy of ulcerative colitis is principally aimed at inhibiting the production of inflammatory mediators and at modulating the immune system. The ideal aim of treatment of ulcerative colitis is to relieve pain, heal the ulcer and delay ulcer recurrence. To date, no drug meets all the goals of therapy, besides existing drugs are relatively expensive. Therefore phytogenic agents have traditionally been used by herbalists and indigenous healers for the prevention and treatment of ulcer (Borrelli and Izzo, 2000). The presence of tannin, alkaloids, saponins, flavonoids, steroids, terpenoids and glycosides in the extracts as reported by Ainooson *et al.*, (2012), may have contributed to the effects of *Trichilia* bark extracts. Presupposing that, *Trichilia* tannins could line the endothelial surface of the mucosa, rendering it less permeable and more resistant to injury. Flavonoids present in *Trichilia* bark would have increased mucosal prostaglandin content, decreasing histamine secretion from mast cells by inhibition of histidine decarboxylase and scavenge free radicals as well as gastric proton pump, inhibition of the lipoxygenase (Borrelli and Izzo, 2000).

Regression or remission of colitis was observed in the excised colon segments of $TMBE_{Et}$ on indo-induced UC, where the normal/naive animal group showed absence of ulcers, the disease control group revealed distorted and swollen mucosa, evident of ulceration and oedema. Pathology then regressed in the $TMBE_{Et}$ treated groups in a dose dependent fashion from few ulcers and epithelialisation of mucosa, evident of healing in the 30 mg/kg $TMBE_{Et}$ group, epithelialisation and mucosal convolution as evidence of pronounced healing in the 100 mg/kg $TMBE_{Et}$ group, and zero ulcers with restored endothelia, evident of complete healing in the 300 mg/kg dose $TMBE_{Et}$ group. The initial injury in this colitis was a relatively bland epithelial necrosis and oedema that variably extended into the lamina propria, submucosa and external muscle layers depending on the concentrations and length of exposure to acetic acid.

Restitution and remission of ulceration in the $TMBE_{Et}$ on AA-induced UC was then clearly manifested in the colonic micrographs of the normal control. Colonic micrographs of the $TMBE_{Et}$ groups on the other hand manifested a dose dependent healing consistent with data for wet colonic weights in such a way that the 30 mg/kg $TMBE_{Et}$ showed mildly eroded and slightly oedematous mucosa, suggesting that healing was slow. The degree of healing was however pronounced in the 100 mg/kg $TMBE_{Et}$ group which showed marked epithelialisation and mucosal convolution, while the 300 mg/kg $TMBE_{Et}$ group, had a restored and highly convoluted mucosa, suggesting that healing had completed. Degree of healing was appreciable in the standard group showing erythematous mucosae with intact submucosa & muscularis.

The study of $TMBE_{Aq}$ has shown that acetic acid-induced ulcerative colitis was associated with macroscopic, microscopic and biochemical changes with remission of mucosal ulceration pointing to a dose dependent respond to *Trichilia* aqueous treatment for both macroscopic and microscopic scores. Earlier phytochemical analysis of the *Trichilia monadelpha* bark extracts showed the presence of tannins, saponins and alkaloids (Ainooson *et al.*, 2012) which could be responsible for the restitution of colonic ulcers. Colonic micrographs of the 30 mg/kg $TMBE_{Aq}$ group manifested ulceration with oedema as evidence of slow healing, but pronounced in the 100 mg/kg $TMBE_{Aq}$ group which showed mucosal columnisation, and the 300 mg/kg dose $TMBE_{Aq}$ group had restored mucosal integrity, depicting appreciable healing. On the contrary, the disease control group had highly serrated mucosae, a manifestation of severe ulceration relative to the normal control which had convoluted mucosae with no evidence of ulceration.

The microscopic evaluation of colonic damage showed increased score in the disease control rats of the $TMBE_{Pt}$ on AA-induced UC, but which decreased in the standard and $TMBE_{Pt}$ treated groups except for the the 300 mg/kg dose $TMBE_{Pt}$ treated group. Micrographs of colons for animals in the 300 mg/kg dose treated group were highly cryptic, associated with suspected toxicity. Evaluation of the colonic damage of the $TMBE_{Ea}$ on AA-induced UC showed higher scores in the 300 mg/kg dose $TMBE_{Ea}$ group as with the disease control rats. There was a decrease in colonic damage score for the standard treated group. Colonic damage notably increased with increasing dose among the *Trichilia* treated groups.

Acetic-acid-induced colitis is an easily inducible model, and the inflammatory mediators profile suggests that the inflammatory phase bears some resemblance to acute human intestinal inflammation (Elson *et al.,* 1995). Mucosa and submucosal inflammation of $TMBE_{Et}$ on AA-induced UC followed initial injury and was associated with activation of arachidonic acid pathways (Elson *et al.,* 1995). Myeloperoxidase and malondialdehyde as inflammation and oxidative stress parameters were measured to ascertain the degree of inflammation and the endogenous oxidative capacities of the experimental animals. A reduction in colonic MPO activity as well as the histological finding of the absence of cellular infiltration following treatment with *Trichilia* may indicate its antioxidant and anti-inflammatory effects in the prevention of acetic acid-induced colitis (Hagar *et al.,* 2007). The antioxidant activity of $TMBE_{Et}$ has been demonstrated in a rapid screening test with TLC. This activity corroboratively showed in the malondialdehyde titres among the *Trichilia* treated groups.

The antioxidant property of $TMBE_{Aq}$ attenuated colitis as shown by the lower serum levels of malondialdehyde among the $TMBE_{Aq}$ treated groups (especially the 100 mg/kg 300 mg/kg treated groups) relative to the standard treated group even though myeloperoxidase activity was poorly curtailed. The reduced colonic tissue contents of lipid peroxidase implied improvement in response to treatment (Hagar *et al.,* 2007). It is therefore reasonable to assume that the $TMBE_{Aq}$ treatment improves colonic oxidative balance in colitic rats, because it was able to reduce the level of MDA, a good indicator of lipid peroxidation and MPO activity, a marker of polymorphonuclear leukocyte accumulation (Paiva *et al.,* 2003).

Mitochondrial oxidative phosphorylation generates most of the ROS and alterations in this metabolic pathway may be related to oxidative stress. Colonic inflammation may produce high levels of oxidants that probably exceed the low antioxidant capacity and lead to epithelial cell disruption (Damiani *et al.,* 2007). TBARS, a marker of lipoperoxidation, had decreased significantly among the *Trichilia* treated rats of $TMBE_{Pt}$ contrary to myeloperoxidase activity.

The results showed that $TMBE_{Ea}$ was unable to reduce the MPO activity associated with acetic acid induce colitis. MPO is a membrane bound haeme enzyme released from neutrophil storage granules following inflammatory stimuli. A reduction in the activity of this enzyme can be interpreted as a manifestation of the anti-inflammatory activity (Paiva *et al.,* 2003), but which was not the case. The increase in MPO activity persisted in $TMBE_{Ea}$ treated rats similar to the disease control group pointing to a failed treatment, corroborative of the colonic damage manifested in the micrographs of animal colons. Malondialdehyde levels among the $TMBE_{Ea}$ treated groups were however appreciable in the 100 mg/kg and 300 mg/kg treated groups similar to the $TMBE_{Pt}$ treatment.

Oedematous colon segments of all the $TMBE_{Et}$ on indo-induced UC weighed heavier than normal colons of the naive group. There however was a reduction in colonic weights correspondent with amelioration of ulceration. The trend was again dose dependent, with an apparent effective dose, beyond which increasing doses of $TMBE_{Et}$ showed no further decrease in colonic weight. Initial epithelial damage is said to be mediated partly by inhibition of the protective prostaglandins PGE1, PGE2, and prostacyclin synthesis. The indomethacin induced model has the advantage of being easily induced, in acute/chronic phases (Jurjus *et al.*, 2004).

Wet colonic segments of all the $TMBE_{Et}$ on AA-induced UC were heavier than normal colons of the naive, pointing to erythema and oedema. Treatment was effective in a dose dependent fashion among the $TMBE_{Et}$ groups and most effective in the standard treated group. This reduction in colonic weights corresponds with amelioration of inflammation and oedema. As expected, there were much heavier colons among the disease control group.

The weight of inflamed colon tissue is considered a reliable and sensitive indicator of the severity and extent of inflammatory response (Paiva *et al.,* 2003). The $TMBE_{Aq}$ significantly reduced the wet weight of distal colon segments and the colon damage score, compared to controls that received the vehicle. Further, it effectively reduced the histological signs of inflammation such as leukocyte infiltration, oedema and tissue injury.

Wet colonic weights reduced in a dose dependent fashion among the *Trichilia* treated groups and most effective in the standard treated group but not the disease control group which had registered much heavier colons, pointing to severe erythema and oedema, correspondent with disease control pathogenesis.

Wet colonic weights of $TMBE_{Pt}$ treated groups increased with increasing dose relative to the standard treated group which registered a marked reduction in colonic weight relative to the naive animals. The disease control group still pointed to severe erythema and oedema. Colonic weights of $TMBE_{Ea}$ treated groups similarly, increased in a dose dependent fashion among the *Trichilia* treated groups relative to the standard treated group which registered a marked reduction in colonic weight comparable to the naive animals. The disease control group on the other hand was reasonably heavy due erythema and oedema.

Animal faecal occult blood of $TMBE_{Et}$ on indo-induced UC showed complimentary evidence of ulceration and bleeding in the disease control and 30 mg/kg $TMBE_{Et}$ animal groups. The trend pointed to a dose dependent pharmacological effect. Luminal bacteria and bacterial products clearly contributed to the inflammatory response. It involves small and large intestines and is associated with extra-intestinal lesions (Elson *et al.*, 1995). Animal faecal occult blood was visible in the low and 100 mg/kg $TMBE_{Et}$ animal groups of $TMBE_{Et}$ on AA-induced UC but regressed relative to the disease control group whose faecal occult blood soared over the experimental period. There was thus a dose dependent effect with the 300 mg/kg dose group responding similarly to the standard treated group.

Faecal occult blood of the $TMBE_{Aq}$ on AA-induced UC differed from the trend of drug activity partly attributable to the illusive faecal stains of the *Trichilia* crude extract that led to positive faecal occult blood test in all $TMBE_{Aq}$ animal groups. This was however not the case in the naive animal group and the standard treated group. Faecal occult blood as an indicator of persistent ulceration increased with increasing dose among the $TMBE_{Pt}$ treated groups.

This was however much severe in the disease control group but marginally decreased in the standard treated group. Faecal occult blood increased with increasing dose among the $TMBE_{Ea}$ treated groups. The trend was much severe in the disease control group similar to the 300 mg/kg dose treated group, but marginally decreased in the standard treated group.

Evidence of recovery was telling in the animal stool consistency examination, with well-formed pellets for all *Trichilia* treated groups similar to stools of naive animals. The disease control group of $TMBE_{Et}$ on indo-induced UC stayed diarrhoea all through the experimental period due to persistent pathology associated with untreated colitis. Diarrhoea associated with acetic-acid induced ulcerative colitis as in the $TMBE_{Et}$ on AA-induced UC is regressive once induction is discontinued (Jurjus *et al.,* 2004). This was evident in the animal stool consistency examination, which showed well-formed pellets for all animal groups similar that of naives.

Stool consistency resulting from acetic-acid induced ulcerative colitis showed well-formed pellets for all animal groups of the $TMBE_{Aq}$ on AA-induced UC. This observation confirms that diarrhoea associated with acetic-acid induced ulcerative colitis is regressive once induction is discontinued (Jurjus *et al.,* 2004). Stool consistency as an indicator of eminent diarrhoea associated acetic-acid induced ulceration showed well-formed pellets for all animal groups of $TMBE_{Pt}$ on AA-induced UC post treatment, even though same could not be said post induction. The presence or absence of diarrhoea was measured for the $TMBE_{Ea}$ on AA-induced UC and which revealed diarrhoea in all animal groups post induction but the naives. However, all the animal groups had demonstrated well-formed pellets for all animal groups post treatment.

General body weights of $TMBE_{Et}$ on indo-induced UC dropped in all the groups but the naive animals, which showed a normal growth pattern. This is presumably attributed to the limited recovery period, since the results looked much closer to the rapidity with which *Trichilia* could relieve ulcerative pain and facilitate healing in the acute phase. In the $TMBE_{Et}$ on AA-induced UC, whose mucosal necrosis and transient inflammation were induced by luminal instillation of dilute acetic acid in a dose-responsive fashion (Jurjus *et al.,* 2004), animal body weights of the *Trichilia* treated groups were consistently similar to those of the standard treated group, showing very minimal reduction relative to the disease control group. The naive animals however showed a normal growth pattern with increased weight.

Induction of colitis by acetic acid in rats as with the $TMBE_{Aq}$ on AA-induced UC is one of standardized methods to produce an experimental model of ulcerative colitis. Several major causative factors in the initiation of human colitis such as enhanced vasopermeability, prolonged neutrophils infiltration and increased production of inflammatory mediators are involved in the induction of this animal disease (Elson *et al.,* 1995). Epithelial injuries were relatively specific reaction to organic acids, because HCl at similar pH did not induce a similar injury (Jurjus *et al*., 2004). The *Trichilia* treated groups showed animal body weights similar to those of the standard treated group, with minimal drop relative to the disease control group. While the naive animals showed a slight increase in body weight, the disease control group had a marginal drop in body weight.

Ulceration as a disturbance of the normal equilibrium caused by either enhanced aggression or diminished mucosal resistance (Borrelli and Izzo, 2000), led to loss of appetite, increased somnolence and loss of weight. With this in mind, body weight loss as a common symptom of UC due to loss of appetite and loss of body fluid (Chung *et al.,* 2007), reduced drastically among the *Trichilia* treated groups of $TMBE_{Ea}$ on AA-induced UC in a severer manner relative to the disease control group. Only the naive animal group showed a slight increase in body weight. The disease control group had a marginal drop in body weight. Animal body weights of $TMBE_{Ea}$ on AA-induced UC were recorded soon after induction of ulceration and just before euthenisation at the end of experimentation and it was found that body weights stayed among the *Trichilia* treated groups in much the same way as in the disease control group. Again, only the naive animals could sustain their body weights as from day 1 of experimentation. The disease control group had also dropped in body weight.

3.5 CONCLUSION

This study could establish a pharmacological basis for the use *Trichilia* bark extract to curtail colonic ulceration and/or inflammation. Using chemical induced colitis models, the results showed that; the aqueous extract was a strong anti-oxidant and highly effective in the treatment of UC. The ethanolic extract followed closely with an appreciable anti-oxidant property and was also an effective treatment alternative for UC, but the petroleum ether and ethyl acetate extracts did not seem to be effective treatment options for ulcerative colitis.

CHAPTER FOUR

4.0 MICROBIOLOGY

Effect of *Trichilia* Bark Extract on Intestinal Microflora of Colitic Rats

4.1 INTRODUCTION

4.1.1 Background Information

The ecology of the human intestinal microflora represents one of the most complex and concentrated group of microorganisms in nature. Adding to the complexity is that, each individual's intestinal ecosystem may have its own distinct characteristics and those characteristics are not uniform over time (O'Sullivan, 2000). The GI tract of an adult human is estimated to harbor about 100 trillion viable bacteria. This is approximately 10 times the total number of cells in the human body, accounting for about 900 g of a body's weight. The density of microorganisms in the gut increases dramatically from 10 - 1,000 CFU/ml in the stomach to 10 - 100 billion CFU/gm in the colon, belonging to as many as 400 different species, and anaerobic bacteria out number aerobic bacteria by a factor of 1000:1. Bacteria have been estimated to constitute 35-50% of the contents in the human colon, profoundly influencing nutritional, physiological and protective processes both directly and indirectly. Gut bacteria directly prevent pathogen colonization by competing for essential nutrients and epithelial attachment sites or by producing antimicrobial compounds such as volatile fatty acids and chemically modified bile acids. Indigenous gut bacteria also create a local environment that is generally unfavorable for enteric pathogens a phenomenon termed Colonization Resistance, otherwise referred to as the barrier effect (Orhage and Nord, 2000).

Our understanding of the role that obligate anaerobes play in human infections is based to a great extent, on observations made in animals. Many of the key observations, such as synergy between microbial species, propensity for abscess formation and response to antimicrobial therapy have been made using various animal model systems. In addition, some of the basic molecular mechanisms underlying the virulence of anaerobes have relied heavily on research using animal models (Onderdonk, 2005)

Table 4.1 Microbial Distribution in the Gastrointestinal Tract

	Number of microorganisms (cfu/ml or cfu/g)				
			-----Small Intestine -----		
Microorganisms	Oropharynx	Stomach	Jejunum	Ileum	Colon
Total Count	10^8 - 10^{10}	0 - 10^4	0 - 10^5	10^4 - 10^8	10^{10} - 10^{12}
Aerobic microorganisms					
Streptococcus	10^6 - 10^8	0 - 10^3	0 - 10^4	10^2 - 10^4	10^3 - 10^5
Enterococcus	rare	rare	0 - 10^2	10^2 - 10^4	10^5 - 10^{10}
Staphylococcus	0 - 10^2	0 - 10^2	0 - 10^3	10^2 - 10^5	10^4 - 10^6
Enterobacteria	rare	0 - 10^2	0 - 10^3	10^2 - 10^7	10^4 - 10^{10}
Yeasts	0 - 10^3	0 - 10^2	0 - 10^2	10^2 - 10^4	10^2 - 10^5
Anaerobic microorganisms					
Peptostreptococcus	10^4 - 10^6	0 - 10^3	0 - 10^3	10^2 - 10^6	10^{10} - 10^{12}
Bifidobacterium	0 - 10^2	0 - 10^2	0 - 10^4	10^3 - 10^9	10^8 - 10^{11}
Lactobacillus	0 - 10^3	0 - 10^3	0 - 10^4	10^2 - 10^5	10^6 - 10^8
Clostridium	rare	rare	rare	10^2 - 10^4	10^6 - 10^9
Eubacterium	10^2 - 10^3	rare	rare	rare	10^9 - 10^{12}
Veillonella	10^3 - 10^8	0 - 10^2	0 - 10^3	10^2 - 10^4	10^3 - 10^6
Fusobacterium	10^4 - 10^8	0 - 10^2	0 - 10^3	10^3 - 10^4	10^6 - 10^8
Bacteroides fragilis	rare	rare	0 - 10^3	10^3 - 10^7	10^{10} - 10^{12}
Prevotella	10^6 - 10^8	0 - 10^2	10^2 - 10^4	10^3 - 10^4	10^4 - 10^5

(Orhage and Nord, 2000)

Quantitative microbiological data indicated that facultative species, such as *E.coli* were responsible for early peritonitis and anaerobes, particularly *Bacteroides fragilis* and *Fusobacterium sp.*, were important for abscess development (Onderdonk, 2005).

Many other investigators have also contributed to our knowledge of the pathogenesis of obligate anaerobes using a variety of animal model systems. Some of these models added information to the fund of knowledge about intra-abdominal infections, while others sought to determine the role of obligate anaerobes from sites other than the GI tract during infections (Onderdonk, 2005). Investigators even began to explore the involvement of anaerobes in chronic inflammatory diseases including hepatitis and arthritis (Kennedy *et al.*, 1999).

It is safe to state that documentation of the role of anaerobes in a variety of human diseases can be attributed to these studies and that anaerobes were no longer considered unimportant members of the human microflora. Many of the observations made in the rat model for intra-abdominal sepsis were verified in other animal species such as guinea pigs and mice, using wound infection models. This included the synergy between *E. coli* and *B. fragilis* in provoking purulent infections (Onderdonk, 2005).

4.1.2 Microbial–Host Interactions in the Intestine

The intestinal epithelium is the interface between the host and microbes, with emerging evidence of a dynamic interchange between these microbes, the epithelium, and the underlying lymphoid cells. Microbial antigens traversing the epithelium are found to be picked up by antigen-presenting cells and presented to both effectors and regulatory T cells in the lamina propria. In healthy animals, antigen-presenting cells have a variety of receptors for microbial products and cytokines such as tumor necrosis factor *a* (TNF-*a*) that activate these cells by means of the nuclear factor-*k*B (NF-*k*B) signaling pathway (Videla *et al.*, 1997).

4.1.3 Methods for Analyzing Colonic Microflora

The 21st century has seen the emergence of numerous molecular approaches for the analysis of different aspects of the human intestinal microflora in conjunction with traditional culture methods. These techniques also have the potential to explore the functionality of certain microbial traits in the intestine. The contribution of these studies to the field of probiotics is the key to providing the necessary scientific substantiation for the efficacy of potential probiotic bacteria on intestinal health (O'Sullivan, 2000).

4.1.3.1 Classical Methods for Analyzing Colonic Microflora

The classical techniques include culture-dependent and culture-independent approaches. Both of which are labour intensive and limited by lack of precision (O'Sullivan, 2000).

4.1.3.1.1 Classical Culture-Dependent Techniques

Isolating organisms from faecal or intestinal material by culturing is the baseline approach for studies on the human intestinal ecosystem. However, this method of characterizing microbes in a natural ecosystem has its shortcomings, because many microbes in different ecosystems cannot be cultivated by standard culture techniques (Ward *et al.,* 1990). Despite this limitation, culture techniques are absolutely essential to obtaining a complete picture of the diversity and role of the intestinal microbes (Palleroni, 1997). This technique involves plating fresh intestinal material on selective or non selective media and incubating at 37°C (O'Sullivan, 2000).

4.1.3.1.1.1 Non-Selective Culture Methods

Non selective media are generally used to estimate total numbers of both aerobic and anaerobic flora. Examples of which include rumen fluid-glucose-cellobiose agar (RGCA) (Moore and Holdeman 1974); modified medium 10 (Wilson and Blitchington, 1996); plate count agar (Alander *et al.*,1997); brucella blood agar (BBA) supplemented with 0.5% sheep blood, 1 mg/ml vitamin K_1 and 5mg/ml hemin (Langendijk *et al.*,1995); and brain heart infusion (BHI) (Ramare *et al.*,1996). It should be emphasized that while these media contain no known selectivity, they do inherently select against some bacteria from the human intestine which has extra requirements (O'Sullivan, 2000).

4.1.3.1.1.2 Selective Culture Methods

Enumeration of specific bacterial genera is generally achieved by plating on selective media. Several selective agents can be used for selective enrichment. The use of these selective agents is thought to inhibit many colonic strains and therefore underestimating the microbial count.

Bacteroides species are the most numerically dominant bacteria and therefore can be isolated without selective agents, however, bile, esculin or antibiotics can be used for selective enrichment for *Bacteroides* species (Engelkirk *et al.,* 1992).

Bifidobacterium is another dominant genus in the intestine and selective media that have been used for Bifidobacteria are: YN-6 (Resnick and Levin, 1981); Pentuey's selective medium (PSM) containing pyruvic acid and naladixic acid (Tanaka and Mutai, 1980); BS1 (Mitsuoka *et al.,* 1965); BIM-25 (Muñoa and Pares, 1988); and Beerens medium (Beerens, 1991). Bifidobacteria selective agents in these media mainly include propionic acid or antibiotics like kanamycin, naladixic acid, paramycin and polymyxin B (Silvi *et al.,* 1996).

Other intestinal bacteria such as lactobacilli are commonly cultured from faecal or intestinal samples using either Rogosa (Difco) or acidified Man Rogosa Sharpe (MRS; Difco) or LAMVAB (Hartemink *et al.,* 1997) media. Clostridia, present particularly in older individuals, can be isolated using novobiocin colistin agar (NCA) and colistin crystal violet agar (CCA) (Fujisawa *et al.,* 1995). Enterococci and fecal streptococci can be isolated using Stanetz-Bartley (SB) medium, also called Bactom Enterococcus Agar (Difco), or oxolinic acid-esculin-azide (OAA) (Audicana *et al.,* 1995). And *Enterobacteriaceae* can be isolated using MacConkey.

All selective media are valuable tools for isolating intestinal microflora, however, they are all not without disadvantages. They are either not absolute selectivity or are toxic against certain strains within the genus. In addition, all culture media may fail to cultivate organisms which are in their physiological state, often termed a 'non-culturable' state. When the culture-dependent technique is used to isolate culturable bacteria from faecal or intestinal samples, it requires confirmatory analysis of the genus and further characterisation of the species or strain, which involves a host of morphological and biochemical tests. The confidence level of the species identification will increase with increasing biochemical tests (O'Sullivan, 2000).

4.1.3.2 Classical Culture-Independent Techniques

Total reliance on culturing could give an uncertain picture, but a number of classical tools have been developed to give valuable insight into the real numbers of microflora in faecal samples (O'Sullivan, 2000). These techniques include direct microscopic analysis (Holdeman *et al.*, 1977) and monitoring specific enzymes or metabolites (Rowland, 1989).

4.1.3.2 Modern Methods for Analyzing Colonic Microbes

4.1.3.2.1 Culture-Dependent Molecular Approaches

The advent of molecular tools like fingerprinting techniques, primarily a DNA based, can be used to reliably identify isolates and their evolutionary relatedness between strains, but this strategy is limited by the extensiveness of the particular fingerprinting database. A major advantage of using a fingerprinting approach for typing microbes is its rapidity and conduciveness to analyse a large throughput of unknown isolates (O'Sullivan, 2000).

4.1.3.2.1.1 Phenotypic Fingerprint Analysis

Phenotypic fingerprints like polyacrylamide gel electrophoresis, fatty acid analysis, bacteriophage typing and serotyping are usually less sensitive and changes in the fingerprint may not necessarily mean a different organism, but rather could be attributed to a change in expression of the particular phenotypic trait. This strategy has been applied for the analysis of two *Bacteroides* species in different human intestines (Corthier *et al.,* 1996).

4.1.3.2.1.2 Genotypic Fingerprint Analysis

The recent development of multiple genotypic fingerprinting methods has been a major advantage for deciphering the complex human intestinal ecosystem. The first molecular method was hybridization with a nucleic acid probe targeted at a specific DNA sequence. This is an elementary fingerprinting technique. The more potentially useful techniques for the study of the human intestinal microflora include; Pulse Field Gel Electrophoresis (PFGE), Ribotyping, Restriction fragment length polymorphism (RFLP) of the 16S rRNA Gene, Multiplex-PCR, Arbitrary Primed (AP) PCR, and Triplet Arbitrary Primed TAP - PCR (Corthier *et al.,*1996).

4.1.3.2.2 Culture-Independent Molecular Approaches

The advent of culture-independent molecular techniques from faecal samples using PCR has in many ways revolutionised the field of microbial ecology (de Vos *et al.,* 1997; Suau *et al.,* 1999; Wilson and Blitchington, 1996). Recently, these molecular techniques have begun to be directed to the human intestine and should unveil many more mysterys of its complex microflora. Generally, faecal samples are first enriched for bacterial cells by differential centrifugation and used directly in PCR, or total DNA/RNA is extracted. The procedure then targets the rRNA genes in a standard PCR. Total RNA can also be used as template if the enzyme reverse transcriptase is also included (O'Sullivan, 2000).

There are limitations with these rRNA based culture-independent techniques, regarding the disparity in the number of rRNA operons in different bacteria. Clearly, an organism with one copy of rRNA genes will be under represented compared to organisms with eight or more copies. The disparity is magnified if rRNA is used as the template for the PCR. Another limitation has to do with the use of universal primers because the primers are not identically homologous to all bacteria and will not amplify all rRNA products with the same efficiency for the amplification of the rRNA product, resulting in a disparity in the biodiversity in favour of those organisms more conducive to PCR with the primers used. To avert this, different sets of primers targeting different universally conserved regions within the rRNA can be used. This culture-independent molecular technique could be run in either of three strategies; cloning and sequencing of individual rRNA genes, separation of individual rRNA products by denaturing gradient gel electrophoresis (DGGE) and checkerboard hybridization with specific probes (Zoetendal *et al.*, 1998). It can also reveal the presence of new isolates, such as ingested probiotic strains that may be present (de Vos *et al.*, 1997).

4.1.4 Laboratory Diagnosis of Enterobacteriaceae

Specimen suspected of containing members of the Enterobacteriaceae are usually inoculated onto two (2) media, a blood agar plate and a selective differential medium such as MacConkey's agar or eosin-methylene blue (EMB) agar. The *differential* ability of these latter media is based on lactose fermentation, which is the most important metabolic criterion used in the identification of these organisms. On these media, the nonlactose fermenter, eg, *Salmonella* and *Shigella,* form colourless colonies, whereas the lactose fermenters form coloured colonies. The *selective* effect of the media suppressing unwanted gram-positive organisms is exerted by bile salts or bacteriostatic dyes in the agar (Betty *et al*, 2007).

An additional set of screening tests, consisting of triple sugar iron (TSI) and urea agar is done prior to the definitive identification procedures (Prescott, 2002). The results of the screening process are frequently sufficient to identify the genus of an organism; however, an array of 20 or more biochemical tests is required to identify the species (O'Sullivan, 2000).

Another valuable piece of information used to identify some of these organisms is their motility, which is dependent on the presence of flagella. *Proteus* species are very motile and characteristically "swarm" over the blood agar plate, obscuring the colonies of other organisms. Motility is also an important diagnostic criterion in the differentiation of *Enterobacter cloacae,* which is motile, from *Klebsiella pneumoniae,* which is nonmotile. If the results of the screening tests suggest the presence of a *Salmonella* or *Shigella* strain, an agglutination test can be used to identify the genus of the organism (Jawezt *et al.,* 2007).

4.1.4.1 Laboratory Diagnosis of *Escherichia coli*

Specimen suspected of containing enteric gram-negative rods such as *E. coli* are grown initially on a blood agar plate and on a differential medium, such as EMB - agar or MacConkey's agar. *E. coli* which ferments lactose forms pink colonies, whereas lactose-negative organisms are colourless. On EMB agar, *E. coli* colonies have a characteristic green sheen (Betty *et al*, 2007). Some of the important features that help distinguish *E. coli* from other lactose-fermenting gram-negative rods are as follows: 1) it produces indole from tryptophan, 2) it decarboxylates lysine, 3) it utilizes acetate as its only source of carbon, and 4) it is motile (Jawezt *et al.,* 2007).

4.1.4.2 Laboratory Diagnosis of *Salmonellae*

In enterocolitis, the organism is most easily isolated from a stool sample. However, in the enteric fevers, a blood culture is the procedure most likely to reveal the organism during the first 2 weeks of illness (Jawezt *et al.*, 2007). Salmonellae form non-lactose-fermenting (colourless) colonies on MacConkey's or erosin-methylene blue (EMB) agar. On TSI agar, an alkaline slant and an acid butt, frequently with both gas and H_2S (black colour in the butt), are produced (Prescott, 2002). *S. typhi* is the major exception; it does not form gas and produces only a small amount of H_2S (Betty *et al*, 2007). If the organism is urease-negative (*Proteus* organisms, which can produce a similar reaction on TSI agar, are urease-positive) the *Salmonella* isolated can be identified and grouped by the slide agglutination test. In cases of enteric fever and sepsis, when the organism is difficult to recover, the diagnosis can be made by detecting a rise in antibody titre (Widal test) in the patient's serum (Jawezt *et al.*, 2007).

4.1.4.3 Laboratory Diagnosis of *Shigellae*

Shigellae form non-lactose-fermenting (colourless) colonies on MacConkey's or EMB agar. On triple sugar iron (TSI) agar, they cause an alkalinization with no gas and or H_2S (Prescott, 2002). Confirmation of the organism as *Shigella* and determination of its group are done by slide agglutination. One important adjunct to laboratory diagnosis is a methylene blue stain of a faecal sample to determine whether polymorphonuclear cells (PMNs) are present, indicating that an invasive organism such as *Shigella, Salmonella*, or *Campylobacter* is involved rather than a toxin-producing organism such as *Vibro cholerae, E. coli*, or *Clostridium perfringens.* Certain viruses and the parasite *Entamoeba histolytica* can also cause diarrhea without PMNs in the stool (Jawezt *et al.*, 2007).

4.1.4.4 Laboratory Diagnosis of *Bacteroides*

Bacteroides can be isolated anaerobically on blood agar plates containing kanamycin and vancomycin to inhibit unwanted organisms (Betty *et al*, 2007). They are identified by biochemical reactions (eg, sugar fermentations) and by production of certain organic acids (eg, formic, acetic and propionic acids), which are detected by gas chromatography, *B melaninogenicus* produces characteristic black colonies (Jawezt *et al.*, 2007).

4.1.4.5 Laboratory Diagnosis of *Pseudomonas aeruginosa*

P. aeruginosa grows as non-lactose-fermenting (colourless) colonies on MacConkey's or EMB agar. It is oxidase-positive (Betty *et al*, 2007). A typical metallic sheen of the growth on TSI agar, coupled with the blue-green pigment on ordinary nutrient agar and a fruity aroma, is sufficient to make a presumptive diagnosis (Prescott, 2002).

4.1.4.6 Laboratory Diagnosis of *Vibro cholerae*

The approach to laboratory diagnosis depends on the situation. For diagnosis of sporadic case, a culture of the diarrhea stool containing *V. cholerae* will show colourless colonies on MacConkey's agar because lactose is fermented slowly. The organism is oxidase-positive, which distinguishes it from members of the Enterobacteriaceae (Betty *et al*, 2007). On TSI agar, an acid-slant and an acid-but without gas or H_2S are seen because the organism ferments sucrose (Prescott, 2002). A presumptive diagnosis of *V. cholera*e can be confirmed by agglutination of the organism by polyvalent Ol or non-Ol antiserum. A retrospective diagnosis can be made serologically by detecting a rise in antibody titre in acute- and convalescent-phase sera (Jawezt *et al.*, 2007).

4.1.4.7 Laboratory Diagnosis of *Campylobacter*

A stool specimen is cultured on a blood agar plate containing antibiotics that inhibit most other fecal flora and incubated at 42 ºC in a microaerophilic atmosphere containing 5% oxygen and 10% carbon dioxide, which favours the growth of *C jejuni.* It is identified by failure to grow at 25 ºC, oxidase positivity and sensitivity to nalidixic acid (Betty *et al*, 2007). A blood culture incubated under standard conditions will reveal the growth of S-shaped, motile, gram-negative rods. Identification of the organism as *C. intestinalis* is confirmed by failure to grow at 42ºC, its ability to grow at 25ºC, and its resistance to nalidixic acid (Jawezt *et al.*, 2007).

4.1.4.8 Laboratory Diagnosis of *Helicobacter pylori*

It can be cultured on the same media as campylobacters (Betty *et al*, 2007). In contrast to *C. jejuni*, it is urease-positive. The "urease breath" test, in which radio-labeled urea is ingested, can also be used. If the organism is present, radio-labeled CO_2 is evolved and the radioactivity detected in the breath (Jawezt, *et al.*, 2007).

4.1.4.9 Laboratory Diagnosis of *Proteus sp.*

These organisms usually are highly motile and produce a "swarming" overgrowth on blood agar, which can frustrate efforts to recover pure cultures of other organisms (Betty *et al*, 2007). Growth on blood agar containing phenylethyl alcohol inhibits swarming, thus allowing isolated colonies of *Proteus* and other organisms to be obtained (Jawezt *et al.*, 2007). They produce non-lactose-fermenting (colourless) colonies on MacConkey's or EMB agar (Betty *et al*, 2007). *Proteus vulgaris* and *P. mirabilis* produce H_2S, which blackens the butt of TSI agar (Prescott, 2002). *P mirabilis* is indole-negative, whereas the other 3 species are indole-positive, a distinction that can be used clinically to guide the choice of antibiotics. These four (4) medically important species are urease-positive (Jawezt *et al.*, 2007).

4.2 METHOD

4.2.1 Drugs and Reagents

Indomethacin granules and tween 80 were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. While Nutrient Agar, MacConkey Agar, Bismuth Sulphite Agar, Peptone media, Triple Sugar Iron, and Citrate were supplied by Oxoid Ltd., Basingstoke, Hampshire, England.

4.2.2 Animals and Experimental Design

Animals were obtained from the Animal House of the Department of Pharmacology, KNUST, fed on commercial pellet diet from GAFCO, Tema-Ghana, and provided with water *ad libitum.* All animal experiments were carried out in accordance with stipulated international standards for laboratory animal use. Thus, maintaining such animals in the animal holding room at 24℃ ± 2℃ with a relative humidity of 70% ± 5˚c and a 12 h light-dark cycle. Wistar rats of comparable age and weight of between 150 g – 200 g were pooled into five groups and housed five (5) in a cage of size 34 x 47 x 18 cm, with wood shavings as beddings. Of the six (6) groups; **GA** = Normal control group (Naive), **GB** = Disease control group (Negative control), **GC** = 30 mg/kg dose group (Parallel treatment), **GD =** 100 mg/kg dose group (Parallel treatment), **GE =** 300 mg/kg dose group (Parallel treatment).

Animals of all groups were starved and given only drinking water for 24 h. The rats were subsequently anesthetized lightly with ether. Indomethacin suspension of 7·5 mg kg^{-1} was then prepared in Tween 80 and administered orally to induce acute colonic ulceration in rats of all groups but the normal control (Elson *et al.,*1995). After the animals recovered from anesthesia, chow pellets and tap water were provided *ad libitum* and ethanolic *Trichilia* bark extract was then administered daily for 5 days at 30 mg/kg, 100 mg/kg and 300 mg/kg doses to simulate earlier experiments on colitis.

4.2.3 Preparation of Extracts

A hydro-alcoholic extract (70% : 30%, v/v) was prepared via cold maceration of powdered *Trichilia monadelpha* bark (TMB). Prior to experimentation, the tincture was vaporised and the concentrated crude extracts, further dried in hot air oven at 60 ^{o}C.

4.2.4 Preparation of Media

4.2.4.1 Chocolate Agar

Chocolate agar was prepared by completely haemolysing 10% fresh sheep blood in hot molten nutrient agar at 80 ^{o}C and employed to distinguish between aerobic and anaerobic bacteria under reduced oxygen (Watson, 2012).

4.2.4.2 Blood Agar

Blood agar on the other hand, was prepared by emulsifying 5% fresh blood in cooled molten nutrient agar at 45 ^{o}C and employed to grow and verify the absence of *Staphylococcus epidermidis and Streptococcus pyogenes* (Kelleher, 2004). All other media were prepared in accordance with manufacturer's manual.

4.2.5 Microbiological Studies

4.2.5.1 Gram staining

Colon swaps were inoculated in nutrient broths for overnight at 37 ^{o}C following which loopful of representative cultures were heat-fixed on slides, stained with ammonium oxalate crystal violet solution for 20 sec and washed gently in water, then with Lugol's iodine solution for 30 sec and decolourized with 95% ethyl alcohol for 5 sec and washed with water. It was subsequently counterstained with 0.5% safranin for 30 sec, washed with water and observed under light microscope for the persistence of the purple and or pink stains (Betty *et al.*, 2007).

4.2.5.2 Agar Plating

Aliquots of 0.2 ml of each broth culture from the nutrient broth inocula were then flooded variously on sterile Nutrient Agar, Chocolate Agar, Blood Agar, MacConkey Agar, and on Bismuth Sulphite Agar under aerobic or anaerobic conditions at appropriate dilution volumes of 1 X 10^{-2} cm^{-3}. Plates inoculated for anaerobes were incubated in an anaerobic condition by the candle-gas-jar approach for 48 h at 37 ^{o}C (Videla *et al.,* 1997).

4.2.5.3 Biochemical Tests

Test tubes containing 10 ml of sterile double strength nutrient broth, peptone media, Koser's citrate media, triple sugar iron, as well as urea slant tubes (Hagar *et al,* 2007) were arranged in a test tube rack and labelled for the purposes of characterization.

4.2.5.3.1 Fermentation Media

Dextrose peptone water incorporated media with Andrade's indicators were inoculated with two loopful of organism suspension and incubated at 35 ^{o}C for 48 hr or more in ambient air for the examination of acid and/or gas production. A pink colouration of the indicator and a collection of gas in the Durham tubes were marked as positive for acid and gas producing organisms respectively (Betty *et al.,* 2007).

4.2.5.3.2 Triple sugar iron (TSI) Test

Inocula of TSI were made by first stabbing a straight inoculating needle to the bottom of each given tube and then streaking the surface. The tubes were loosely capped and incubated at 37 ^{o}C for 24 hours to determine which sugar is being utilized in order to identify the suspected organism as *E. coli*, *Salmonella sp.* and/or *P. aeruginosa* (Betty *et al.,* 2007).

4.2.5.3.3 Hydrogen Sulphite Production

Brewer's media were prepared to allow for the growth of anaerobes (Betty *et al.*, 2007), and with suspended lead acetate impregnated filter papers serving as indicators to test the production of hydrogen sulphite gas. Organisms that are capable of degrading sulfhydryl containing amino acids like cysteine and cistine evolved H_2S gas which then reacted with the lead acetate paper to give black precipitates as indication of positive tubes (Jawetz *et al.*, 2007).

4.2.5.3.4 Urea Hydrolysis Test

Urea agar slants were inoculated and incubated at 37 ^{o}C for 24 h to distinguish *Salmonella sp.* from *Proteus sp.* (Murray *et al*, 2002). Positive sample changed the urea agar from orange to magenta, indicative of *Proteus vulgaris* (Betty *et al.*, 2007).

4.2.5.3.5 Koser's Citrate Slant Agar

Koser's citrate slants were inoculated with straightened wire as described by Cowan and Steel, (1970). The inocula were then incubated 35 ^{o}C for 48 h or more to determine the ability of organisms to breakdown sodium citrate in order to utilize citrate as sole source of carbon (Betty *et al.*, 2007). *Klebsiella pneumoniae* and *Proteus mirabilis* are examples of citrate positive organisms. *Escherichia coli* and *Shigella dysenteria*e are citrate negative (Watson, 2012).

Each inoculum was done in triplicates in order to validate the microbial activity of the *Trichilia* ethanolic extract as basis for subsequent experimentation on infectious colitis.

4.2.6 Statistical Methods

The study was qualitative and descriptive, as such the results was tabulated and illustrated graphically, comparing data the animal groups simultaneously.

4.3 RESULTS

4.3.1 Agar Plates

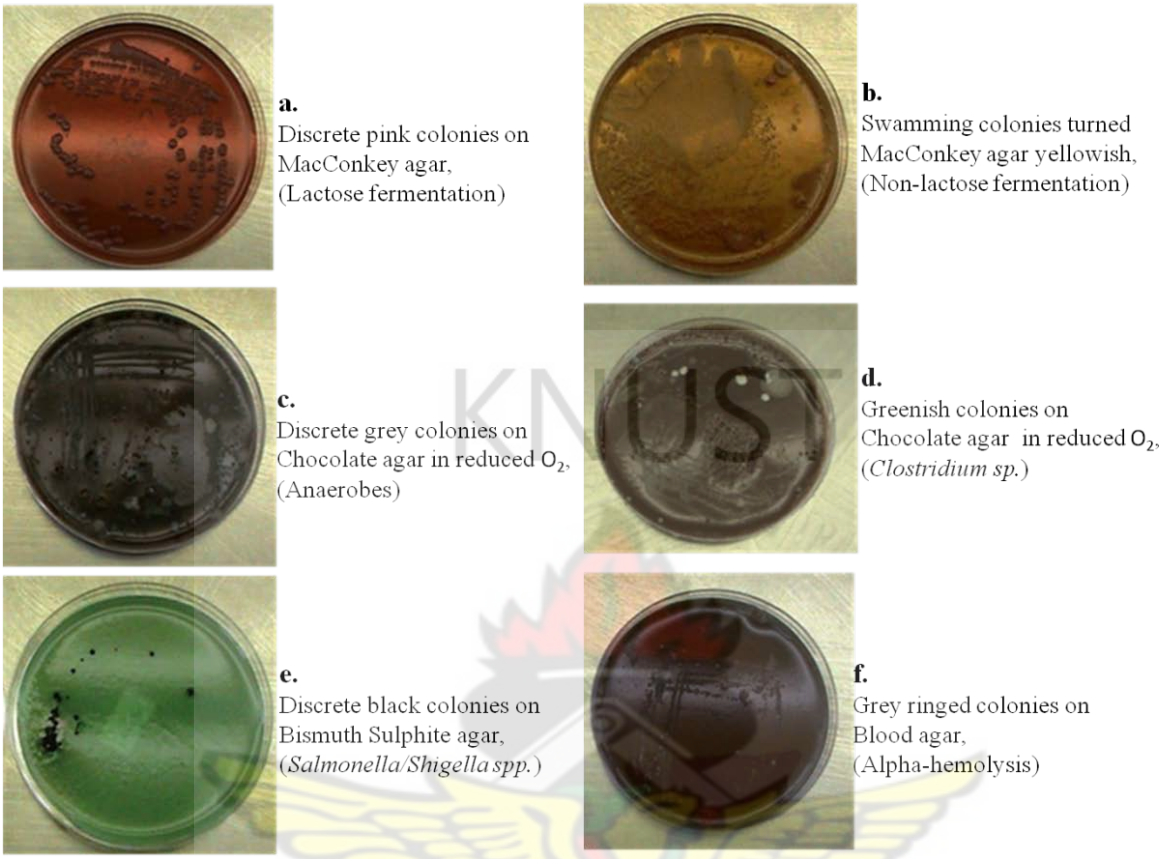


Figure 4.1 Representative Agar Plates

4.3.1.1 Nutrient Agar

Nutrient agar plates incubated at 37 ^{o}C for 24 h had pure colonies reflective of the microbial populations from respective faecal samples for the purposes of biochemical tests.

4.3.1.2 Chocolate Agar

Chocolate agar inocula were positive for all plates incubated under both aerobic and anaerobic conditions, indicative of the presence of both anaerobic and/or facultative bacteria in all animal groups. The aerobic cultures showed varied bacterial colonies for groups GA, GB and GC. Meanwhile, the presence of *Clostridium sp.* in these groups was evident by observable greenish colonies on chocolate plates cultivated under reduced oxygen tension (Fig. 4.1d).

4.3.1.3 MacConkey Agar

Lactose fermenting gram negative Enterobacteria like *Escherichia coli* which occurred in faecal samples of groups GA, GB, GC and GD but not GE, grew on MacConkey agar and with their characteristic acid production turned the entire media red (Fig. 4.1a), with *E. coli* producing pink to red colonies. The non-lactose fermenters were observed in replicates of only GE, and found to have varied the media yellowish to light pink in color. Growths in this group showed colorless colonies (Fig. 4.1b).

4.3.1.4 Haemolysis on Blood Agar

Based on the ability of an organism to break down haemoglobin in red blood cells, only the alpha hemolytic growths were observed in groups GA, GB, GC and GD, indicating that *E. coli* but not *Staphylococcus epidermidis and Streptococcus pyogenes* was occurring in those groups as shown on Tab. 4.2 and Fig 4.1f.

4.3.1.5 Bismuth Sulphite Agar

Bismuth sulphite agar is a selective medium suitable for the cultivation and selective identification of *Salmonella* and/or *Shigella spp.* Thus plating on bismuth sulphite agar was purposeful and black discrete colonies observed in samples of groups GB, GC, GD and GE after 48 h of incubation indicated the presence of *Salmonella* and/or *Shigella spp.* as shown in Fig 4.1e. Meanwhile, the rabbit eye colonies surrounded with black zones as observed on plates of GC, GD and GE in the first 18 h after incubation were indicative of the presence of *Salmonella sp.,* therefore, that plates GB were of *Shigella sp.* The absence of growths on plates of group GA indicated the absence of *Salmonella* and *Shigella spp.* (Tab. 4.2 & Fig 4.2)

Table 4.2 Colonic Microbial Characterization and Identification

Group	GA					GB					GC					GD					GE				
Rat ID	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Isolates	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -	+ -
PRESUMPTIVE																									
Gram + Bacilli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Gram – Bacilli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aerobes/ Facultative	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose Fermenters	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
N/Lactose Fermenters	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
ISOLATES																									
Bacteroides sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Clostridium sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Escherichia coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Eubacterium sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Peptustreptococcus sp.	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Proteus sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp.	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Salmonella sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Shigella sp.	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Present,

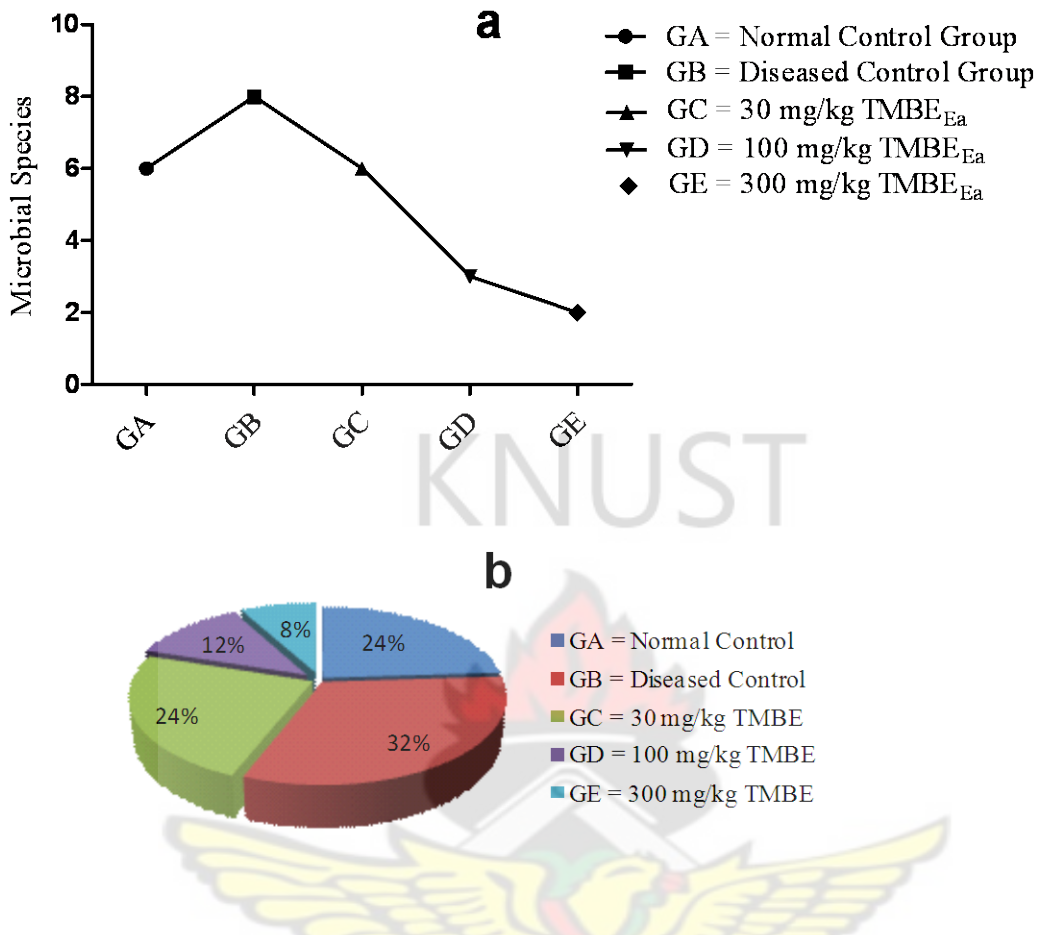


Figure 4.3: Microbial Distribution among Animal Groups

4.3.2 Biochemical Tests

4.3.2.1 Fermentation Media

Peptone water incorporated with Andrade's indicator was used to determine the ability of organisms to ferment specific carbohydrates in the basal medium leading to acid and/or gas production. The change of bromocresol purple indicator to yellow with collection of gas in the Durham tubes in groups GA, GB, GC and GD marked as positive for acid and gas producing *E. coli* as evident in Fig. 4.4c

4.3.2.2 Triple sugar iron Test

Triple sugar iron slants were employed to ascertain whether the gram-negative Bacilli fermented glucose and lactose or sucrose to give out H_2S. The presence of *P. aeruginosa* in GB and GC were implied of alkaline slant without a change in the butt. Slant tubes of GC, GD and GE remained alkaline but with acid butt, indicating a glucose only fermentation of *Salmonella sp.* The presence of *E. coli* in GA, GB, GC and GD were marked by acid slant, acid butt.

4.3.2.3 Hydrogen Sulphite Production Test

The *E. coli* encounted in groups GA, GB, GC and GD produced hydrogen sulphite gas by degrading sulfhydryl containing amino acids like cysteine or cystine, which then reacted with the iron impregnated filter paper to give black precipitates as in Fig 4.4d & 4.4e below

4.3.2.4 Urea Hydrolysis Test

Urea hydrolysis test was carried out according to the Christensen's Method to distinguish between *E. coli* from *Proteus sp.* Both bacteria being lactose fermenters, the urease production test distinguished *P. vulgaris* as positive slants of all faecal samples for all animal groups, by varring the urea agar from orange to magenta (Fig. 4.4).

4.3.2.5 Koser's Citrate utilization test

A Koser's citrate utilization test was carried out to determine if an organism can use sodium citrate as its sole source of carbon and so cause the pH of the medium to alkalinised from green to blue. In this, majority of the test tubes were negative, an indicating the dominance of *E. coli.*

In all, *Bacteroides sp., Clostridium sp., Escherichia coli Enterobacter sp., Proteus sp., Peptostreptococcus sp., Pseudomonas sp., Shigella sp.* and *Samonella sp.* were identified. Out of which 6 species occurred in GA, the normal control group, representing 24%. Relative to the Normal control, the disease control (GB) manifested severe microbial infestation recording up to 8 organisms representing 32%, the highest percentage among the groups. Meanwhile, groups GD and GE, the medium and high dose *Trichilia* treated were identified with 3 and 2 microbes, representing 12% and 8% respectively as shown in Fig 4.3b

The isolates were such that; only *Samonella sp.,* and *Proteus sp.,* were found in the 300 mg/kg dose treated group, *Escherichia coli., Samonella sp., Clostridium sp.,*and *Proteus sp.,* in the 100 mg/kg. But the 30 mg/kg dose and the disease control groups were heavily infested with *Bacteroides sp., Clostridium sp., Escherichia coli Enterobacter sp., Proteus sp., Pseudomonas sp., Shigella Sp.,* and/or *Samonella sp.* The normal control on the other hand was identified with typical ruminant colonic flora such as *Bacteroides sp., Clostridium sp., Escherichia coli Enterobacterium sp., Proteus sp.,* and *Peptostreptococcus sp.,* all of which has been clearly illustrated in Fig 4.2 in conformity with Tab 4.2.

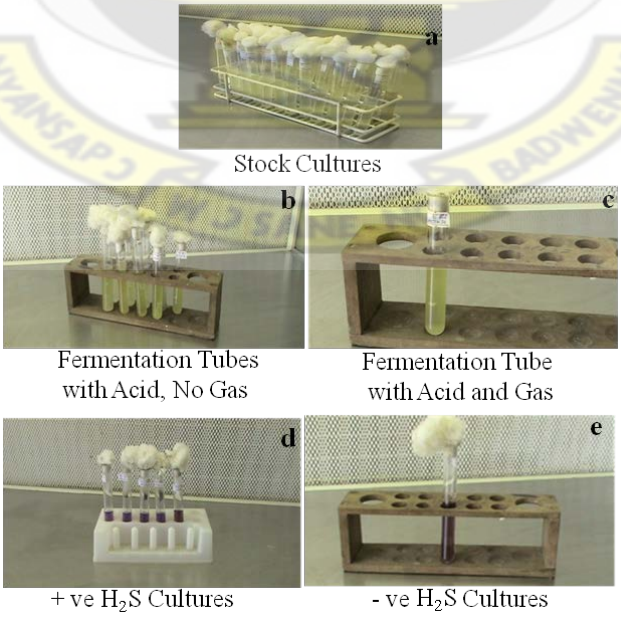


Figure 4.4 Representative Biochemical Tubes

4.4 DISCUSSION

The 8% and 12% microbial infestation among animals of the 300 mg/kg and 100 mg/kg *Trichilia* treated groups was a reflection of the anti-microbial role of *Trichilia monadelpha* as suggested in the Ghana Herbal Pharmacopoeia (Busia, 2007). Complementary to the earlier findings, the 24% and 32% occurrence of colonic microbial infestations in the 30 mg/kg and disease control groups goes to suggest a dose dependent anti-microbial activity.

Meanwhile, if microflora benefits the host by increasing resistance to new colonization as well as by protecting against the overgrowth of already-present potentially pathogenic organisms (Todar, 2008), then increased activity against microflora by *Trichilia monadelpha* as seen with the high and medium dose treatments could have varied implication to the host animal. Moreover, the metabolic role of such microflora has been claimed to be similar to that of the liver (Orhage and Nord, 2000), suggesting that in their absence as was the case among animals of the 300 mg/kg and 100 mg/kg *Trichilia* treated groups, much is lost in the form of micronutrient supply.

Though accurate characterization of the bacterial population of the human faecal flora represents one of the most complex and ambitious studies ever undertaken by bacteriologists (Moore, 1994), it was obvious from the chocolate agar plates that both anaerobic and/or facultative bacteria were present in all samples of all animal groups in contradiction with Moore's (1994) assession that, strict anaerobic bacteria are now recognized as the predominant components of the intestinal microflora in humans and mammals.

The aerobic cultures showed varied facultative bacterial colonies especially of group GA, GB and GC, some of which were found to include; *Escherichia coli*, *Proteus mirabilis*, and *Streptococcus faecalis*, while the anaerobic cultures were made of *Bacteroides* and *Peptostreptococcus ssp.* The aerobes colinizes the vaginal and anal orifices, producing an acidic medium that prevent the colonization of pathogens, as such, its presence in groups GA, GB and GC could be protective against infectious colitis. But the increase in acidity on the other hand could incite inflammatory pain on the already colitic colon (Todar, 2008).

Meanwhile, the presence of gram positive *Clostridium spp.*, colonizers of the bowel, in groups GA, GB and GC could lead to pseudomembranous colitis as with antibiotic induced diarrhoea (Todar, 2008). Presumablly, the antimicrobial activity of *Trichilia* must have suppressed the growth of *C. difficile* in the high dose treated groups GD and GE, which would otherwise multiply to produce exotoxins A to cause an outpouring of fluid, resulting in watery diarrhoea and exotoxins B to cause damage to the colonic mucosa, leading to pseudomembrane colitis.

Following the ability of an organism to break down haemoglobin or red blood cells, 3 groups of microorganisms are usually characterized as: Alpha-hemolytic organisms, growing on blood agar with a characteristic green to light-brown halo seen around the colonies as they partially break down haemoglobin leaving a green pigment (biliverdin). Beta-hemolytic organisms, on the other hand will completely break down haemoglobin in blood agar leaving clear zones around the colonies. These bacteria are known to produce "beta-haemolysin" (streptolysin O or S), which lyses red blood cells in the medium. Meanwhile the third group, gamma-haemolytic organisms, do not produce haemolysin, as such are not able to break down the hemoglobin component in blood agar and so their growth had no effect on the medium (Kelleher, 2004).

On this basis, blood agar was employed to differentiate members of the genera *Staphylococcus*, *Streptococcus* and *Enterococcus* (Watson, 2012). It was found that all the plates were characterised by alpha-hemolytic colonies, indicating the presence of *E. coli*, the agent most frequently associated with "traveller's diarrhea", in all the samples (Kelleher, 2004).

Having inhibited the growth of gram-positive bacteria by the crystal violet in MacConkey agar, the lactose fermenting gram negative Enterobacteria like *Escherichia coli* occurring in faecal samples of groups GA, GB, GC and GD but not GE, grew unrestricted. Though a notable flora, they are frequently passed out with faecal matter, and their proliferation could lead to intestinal and urinary tract infections. As such, animal handlers ought to be mindful of cross infections (Todar, 2008). The non-lactose fermenters like *Proteus sp.* and *Salmonella sp.* occurred in replicates of only group GE, the 300 mg/kg *Trichilia* treated group.

Bismuth sulphite plates of groups GB, GD and GE were positive for *Salmonella* and *Shigella spp.* GD and GE but GB also revealed rabbit eyed colonies after 18 h of incubation, characteristic of *Salmonella sp.* and so, animals of these groups could suffered metastatic abscesses from the *Salmonella* species, an enterocolitis which is characterized by an invasion of the epithelial and subepithelial tissue of the intestines. *Shigella*, the most effective enteric pathogens occurring in GB, the diseased untreated group, on the other hand could have caused dysentery (a human disease). Therefore, animals from GB must have suffered shigellosis while those from GD and GE, the 100 mg/kg and 300 mg/kg dose *Trichilia* treated, suffered salmonellosis. *Salmonella* and *Shigella spp.* are both facultative gram negative bacilli, the former flagellated**,** while the later, non-motile.

In the specific carbohydrates fermentation analyses, a yellow colouration with collection of gas in the Durham tubes in groups GA, GB, GC and GD marked as positive for acid and gas producing *Escherichia coli* (Betty *et al.*,2007), which could have caused watery, non-bloody and self-limiting diarrhea (traveller's diarrhoea, or "turista"). It could however result in a dysenterylike syndrome characterized by bloody diarrhoea, abdominal cramping, and fever similar to that caused by *Shigella* (Jawezt *et al.,* 2007). Slant tubes of GD and GE, the 100 mg/kg and 300 mg/kg dose *Trichilia* treated, on the other hand remained alkaline but with acid butt, indicating a glucose only fermentation of *Salmonella sp*. The virulent gram negative and opportunistic pathogen, *Pseudomonas aeruginosa,* as shown in the triple sugar iron slants for samples of groups GB and GC, the disease untreated and 30 mg/kg *Trichilia* dose groups, had varied implications especially for nosocomial infections (Todar, 2008). *Shigella*, *E. coli*, *Salmonella* as known causative agents of bloody diarrhoea were found to be sensitive to *Trichilia* at higher doses of 100 mg/kg and 300 mg/kg.

4.5 CONCLUSION

The study was conclusive on the anti-microbial role of ethanolic *Trichilia* bark extract, and that its activity increased with increasing dose to the detriment of colonic microflora and could be effective against infectious colitis.

CHAPTER FIVE

5.0 WOUND HEALING

Effect of *Trichilia* Bark Extracts on Excision Wound Healing

5.1 INTRODUCTION

5.1.1 Background Information

Wound disorders present a serious clinical problem and are likely to increase since they are associated with diseases such as diabetes, hypertension, and obesity (Prathiba *et al.*, 2001). The healing of skin wounds progresses through sequential and overlapping phases of inflammation, granular tissue formation, re-epithelialisation and remodelling (Fig 5.1). Each is directed by a complex coordination and interaction of several cell types contained within the wound. Native skin cells such as fibroblasts, keratinocytes, and vascular endothelial cells are also intricately involved in these processes (Frank and Kampfer, 2003). Malnutrition has been observed to profoundly influence wound healing at multiple points in the phases of wound repair. Protein malnutrition or vitamin C deficiency directly inhibits collagen synthesis and deposition, leading to a retardation of the healing process. Wounds are defined as chronic when they heal with a significant delay, usually over a period of more than 2 months. The morbidity associated with delayed wound healing imposes an enormous social and financial burden on the health care system (Frank and Kampfer, 2003).

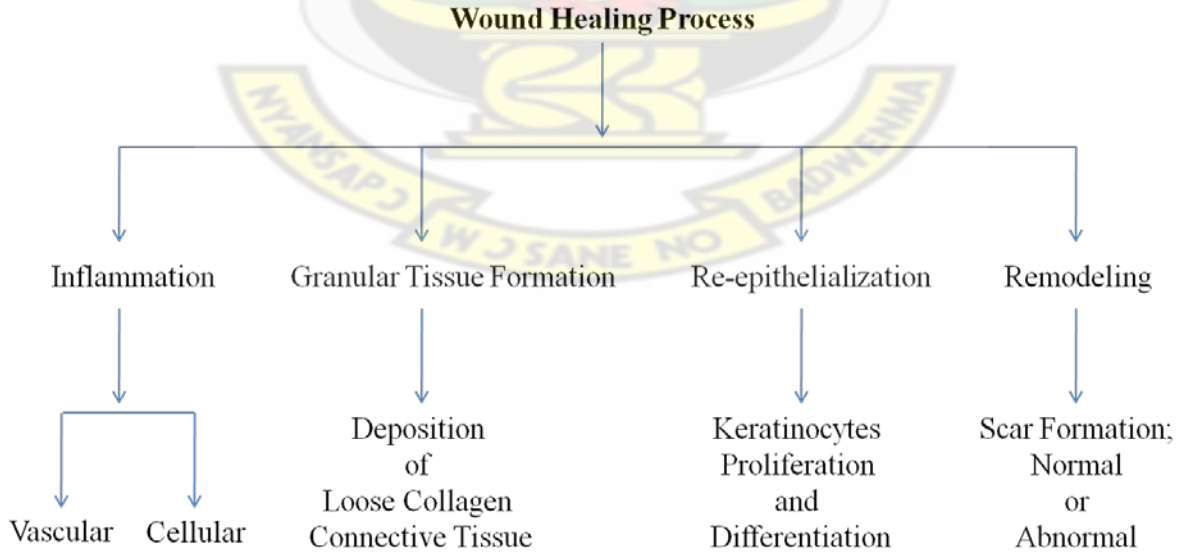


Figure 5.1: Normal Wound Healing Process; Adapted (Prathiba *et al.*,2001).

5.1.2 Inflammatory Mediation and Regulation of Wound Healing

5.1.2.1 Basic Fibroblast Growth Factor (bFGF)

Basic fibroblast growth factor (bFGF) is another substance mainly involved in angiogenesis. It is sequestrated and protected by binding with heparan sulphate which gives stability to bFGF rather than free bFGF. This binding also gives the necessary conformation for optimal interaction with the cell-surface receptors (Prathiba *et al.*,2001).

5.1.2.2 Proteoglycans

The skin which acts as a barrier between the organism and its environment is the most affected organ following an injury. Recent studies have shown that Proteoglycans are synthesized by all types of mammalian cells and that specific Proteoglycans may be responsible for specific functions of the skin layers, because Proteoglycans give a particular structural identity to the layers by sorting out specific cell types to migrate and/or to be retained at the specific layers (Prathiba *et al.*,2001).

5.1.2.3 Hyaluronic acid (HA)

During the inflammatory phase, intact hyaluronic acid (a member of glycosaminoglycan) in the blood clot of wounds helps in the physical stabilization of the matrix. It also stimulates cell infiltration/migration, and controls the degradation of fibrin, thus acting as regulator molecules of the wound healing process. Hyaluronic acid (HA) fragments stimulate both angiogenesis and phagocytosis. It is also a major component of early granulation tissue and creates an environment for cell movement. Higher level of HA persists in the foetus, but falls quickly back to normal in the adult. Hence it has been suggested that the prolonged presence of HA in a wound may account for the scarless repair in the foetus, clearly indicating that HA helps in scarless healing and if suitable levels are maintained in adults during wound healing, scar formation can be prevented.

5.1.2.4 Collagen Fibre

Unlike the normal skin, the collagen bundles in keloids are arranged in a haphazard manner. The improper orientation of collagen bundles in keloids may be attributed to changes in the glycosaminoglycan (GAG) levels, which have certain regulatory functions influencing collagen fibre formation and the three-dimensional organization of collagen. A variety of conditions such as interaction of collagen with proteoglycans are postulated to be important in fibrillar architecture. The elevated level of GAGs in keloid collagen, prevent the removal of water molecules and may result in decreased lateral growth. Due to the water retaining or absorbing capacity, GAGs swell in solution and occupy a large volume (Prathiba *et al.*,2001).

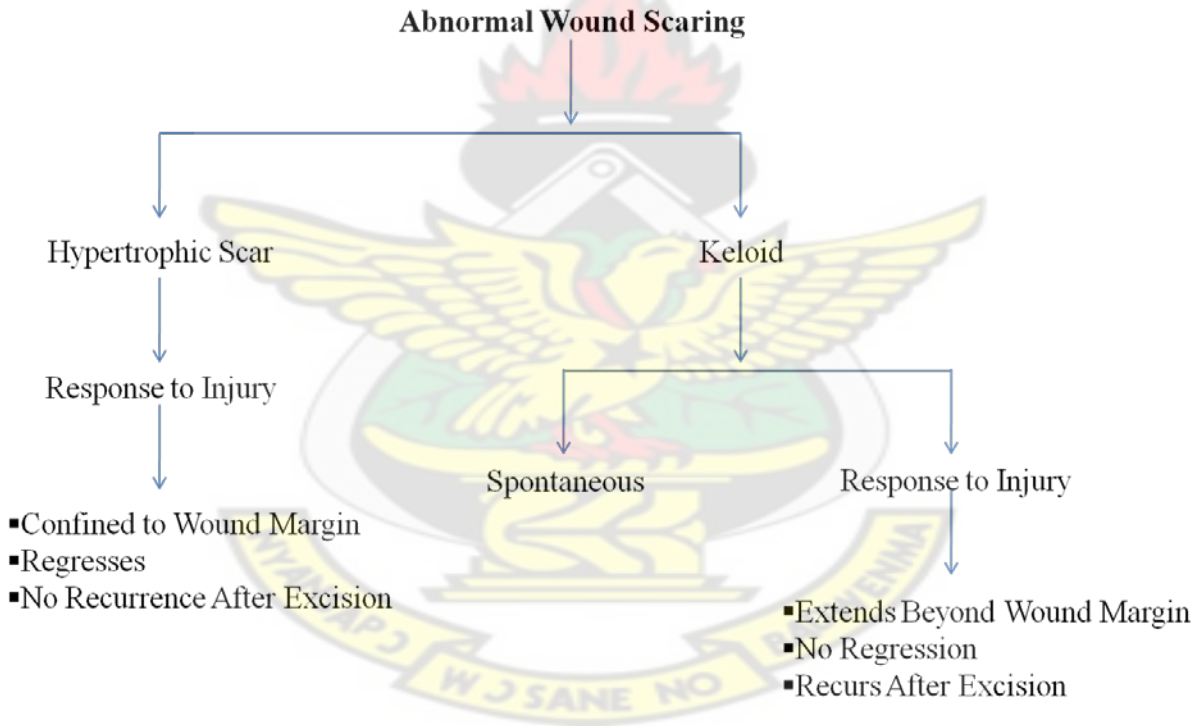


Figure 5.2: Abnormal Wound Healing Process (Prathiba *et al.*,2001).

5.1.3 Experimental Models of Wound Healing

5.1.3.1 Tape Abrasion Skin Injury

Tape abrasion is perhaps the simplest method of creating an epithelial injury. Once hair is depilated and the skin is disinfected, adherent tape could be applied to the skin and then quickly removed with a quick stripping motion moving nearly parallel to the skin, thereby removing the top layers of skin cells. This can be repeated several times until the desired depth of injury is attained. The advantage of this method is that it is inexpensive and very simple to perform. However, the injury is limited to the epidermis, and most often basal cells will be left intact (Paddock *et al.*,2003).

5.1.3.2 Suction Blisters Skin Injury

This technique involves the application of vacuum suction to skin for a period of time ranging from several minutes to an hour or more. It involves slow separation of the epidermis and dermis at their interface followed by fluid filling the intradermal space (Paddock *et al.*,2003).

5.1.3.3 Water Scald Burns

Constant temperature water scald burn models have been created in several species including mice, rats and pigs. Typically, the skin is shaved, and in the case of mice or rats, the animal is placed in a tube-like structure that contains a cut-out area that exposes only a fixed area of the dorsal skin. The unit is then partially submerged in a constant temperature water bath for a fixed time period. In approximately 1 to 2 h a blister will form over the burn, which can be de-roofed to expose the wound. To create a uniform depth of scald injury, it is important to halt the burn process soon after stoppage, which is usually accomplished by applying ice-cold water to the scald. One difficulty in utilizing this technique is creating watertight structures that hold the rodent and prevent scalding of tissue past the intended site (Paddock *et al.*,2003).

5.1.3.4 Ischemic Wound Healing

Clinical observations suggest that persistent tissue ischemia in the vicinity of a wound is an important underlying feature of chronic wounds that severely impairs the healing process. Prolonged ischemia subjects wounds to inflammation, infection, and necrosis and is a significant contributor to delayed healing (DiPietro and Burns, 2003).

5.1.3.5 Dead-Space Wound Model

Subcutaneous Sponge model is one of dead-space models used for studying granulation and reparative tissue in-growth. As opposed to the steel wire mesh cylinder model, it does not have a lag phase and tissue grows within its interstices shortly after implantation. The polyvinyl alcohol (PVA) sponge model provides a relatively biologically inert substance that may be implanted subcutaneously and into which all phases of the healing process are expressed. If properly employed, it can provide information on almost all phases of wound healing, making it a powerful tool for the study of wound healing (DiPietro and Burns, 2003).

The model is best used for acute studies because it begins to elicit foreign body reaction with giant cell accumulation and fibrosis after about two weeks in rats and four weeks in mice. One of the main uses of the PVA sponge implants is to assess reparative wound collagen accumulation by measuring hydroxyproline (OHP) content in sponge acid hydrolysates (DiPietro and Burns, 2003).

5.1.3.6 Incision Wound Healing

The nature and mechanism of incision wound healing has been and continues to be of interest to clinicians and wound biologists. As does any type of injury, incision wounds alter the homeostatic state of the organism and trigger a sequence of events that constitutes three typical pathological phases. In the acute inflammatory phase, infiltrating phagocytes protect the wounded tissue from infection and remove necrotic debris (DiPietro and Burns, 2003).

Within 24 h of wounding, the first inflammatory cells, neutrophils, appear at the margins of the incision and move toward the fibrin clot and fills the narrow incision space immediately after wounding. On day 2, the basal cells of the epidermis demonstrate mitotic activity, migrate, and grow along the cut margins. By day 3, granulation tissue progressively grows into the wound cleft. Collagen fibers also appear at the wound margins. Neutrophils are largely replaced by macrophages during this time, and at about day 5, neo-vascularisation is maximal in the granulation tissue, and abundant collagen fibrils begin to bridge the incision. Meanwhile, the epidermis recovers its normal thickness with progressive keratinisation. During the second week, the inflammatory infiltrates have largely disappeared. Clinically the wound's appearance changes from pink to pale, suggesting continued collagen accumulation and fibroblasts proliferation, as well as the regression of vascular channels (DiPietro and Burns, 2003).

Meanwhile, it is difficult to assess wound healing in incision wounds, since breaking strength measurements reflect only one aspect of healing (DiPietro and Burns, 2003).

5.1.3.7 Excision Skin Wound

5.1.3.7.1 Partial-Thickness Skin Injury

A partial-thickness skin injury can be simply defined as a wound that extends completely through the epidermal layer and only partially through the dermal layer. Epithelial cells that line hair follicles, sweat and sebaceous glands extending into the deep dermis remain viable after a partial-thickness injury. These epithelial cells proliferate and migrate onto the surface of the wound, where they differentiate into epidermal keratinocytes (Paddock *et al.*,2003).

5.1.3.7.2 Full-Thickness Skin Injury

The model of excision skin wounding in mice can be used to assess molecular, cellular, and tissue movements in mice. It represents an animal model that provides access to investigate complex tissue movements associated with repair such as haemorrhage, granulation tissue formation, re-epithelialisation, and angiogenic processes. Moreover, the model can be easily adapted from a basic experimental model to one that deals with more detailed questions of interest (Paddock *et al.*,2003).

5.2 MATERIALS AND METHOD

5.2.1 Drugs and Reagents

For the purpose of this study, a tube of silver Sulphadiazine cream (Silverzine) and emulsifying ointment were acquired from Ernest Chemist, Accra, Ghana, Test drugs (*Trichilia* fractions) were constituted into 3%, 10% and 30% for wound healing (WH).

5.2.2 Animals and Experimental Groupings

Experimental animals were obtained from the Animal House of the Department of Pharmacology, KNUST, upon approval by the Faculty Ethics Committee and in compliance with international standards for laboratory animal use. All such experimental animals were maintained in an animal holding room at 24℃ ± 2℃ with a relative humidity of 70% ± 5˚c and a 12 h light-dark cycle, fed on commercial pellet diet from GAFCO, Tema-Ghana, and provided water *ad libitum.*

The wounding experiments were commenced by hypnotising Wistar rats of comparable age and weight of between 150 g – 200 g and subsequently removing the fur of their whole back skin. Six groups of experimental animals were housed five (5) in cage of size 34 x 47 x 18 cm for each experimental set-up, with wood shavings as beddings. Of the six (6) groups; **GA** = Normal control group (Naive), **GB** = Disease control group (Negative control), **GC** = Standard control group (Positive control), **GD** = 3% dose group (Parallel treatment), **GE =** 10% dose group (Parallel treatment), **GF =** 30% dose group. All animals were dorsally wounded then fed with normal animal feed alongside an appropriate dressing (Parallel treatment).

5.2.3 Wounding Process

The excision wound model which was adopted, and as suggested by Frank and Kampfer, (2003) is envisaged to provide access to investigate complex tissue movements associated with repair such as haemorrhage, granulation tissue formation, re-epithelialisation and angiogenesis.

The choice of circular wounds was because they have a larger and even ratio of total wound area to wound edge which is symmetrical to the wound centre and repair of injured skin areas required coordinated cellular movements to restore epidermal, dermal, and subcutaneous tissue structures (Paddock *et al.*, 2003). The process was initiated by hypnotising the animals with pentobarbitone and skinning. The wounding was then done with a pair of scissors to reach and remove the epidermal, dermal, and subcutaneous layer including the panniculus carnosus by excising skin areas (about 50 mm in diameter) from the backs of the animals. Literature indicates that wounded animals will cope well as it did happen (Frank and Kampfer, 2003). Accordingly, repair of the injured skin required coordinated cellular movements to restore epidermal, dermal, and subcutaneous tissue as expected of the *Trichilia* bark creams.

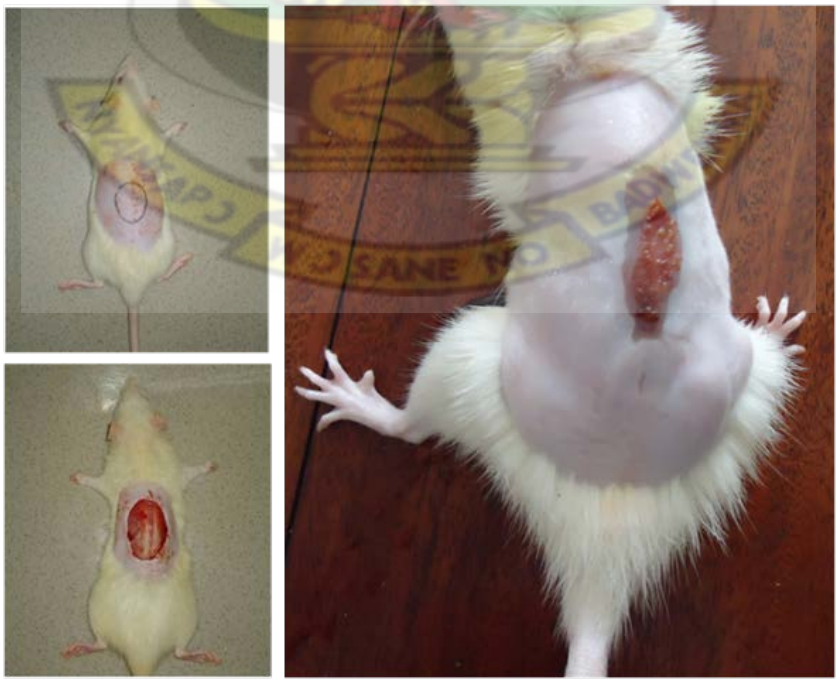


Figure 5.3: The Wounding Process

5.2.4 Wound Assessment

Wound diameter (Wd) was measured as a nominal value of the width of each excised wound on days 1, 3, 5, 7, 9 and 13 respectively.

Wound size (Ws) was then computed as:

5.3 RESULTS

5.3.1 Effect of TMBE on Change in Wound diameter (ΔWd)

Wounds of both vehicle control and aqueous extract ($TMBE_{Aq}$) dressings had enlarged on day 1 post wounding, an indication of progressing pathology due to acute inflammation (See Fig 5.4a & b). By day 3, the acute inflammatory situation had receded. All other groups including the standard control group did not exhibit an increase in wound width on day 1, an indication of effective drug intervention against acute inflammatory responses. The Bonferroni's post-tests revealed a significant difference in the change in diameter for the $TMBE_{Aq}$ on day 13 for the standard group at $P < 0.001$ and $P < 0.05$ for the 3% $TMBE_{Aq}$ dressed group. Interestingly, the 10% $TMBE_{Aq}$ dressing showed significant difference in ΔWd on day 7 at $P < 0.05$ and a significant difference on day 13 at $P < 0.001$ as shown in Fig 5.4a. The trend of healing is clearly manifested in the total Change in Wound diameter (ΔWd) with all treatment groups showing significant difference at $P < 0.001$ against the vehicle dressed. The 3% and 30 % but the 10% $TMBE_{Aq}$ dressed, were significantly different at $P < 0.01$ against the standard silverzine dressed (See Fig 5.4b).

Trichilia bark extracted with ethanol ($TMBE_{Et}$) had a relatively higher activity on excision wounds (ExW) and could be a preferred treatment. Albeit, wounds were generally wider among the ethanolic extract treated groups from onset, reduction in wound diameter was slow, constant and in a dose dependent fashion relative to the standard silver dressing. But in relation to the vehicle control group, the $TMBE_{Et}$ dressings demonstrated some appreciable reduction in wound size by day 13 as shown in Fig 5.4c & d.

Evidence showed that *Trichilia* bark extracted with ethanol as an organic solvent was slow but had maximal effect at the end of experimentation. The 3%, 10% and 30% dressings of $TMBE_{Et}$ all showed no significant ΔWd ($P > 0.05$), while the standard had $P < 0.001$ on day 13 of treatment indicating a significant ΔWd at $P < 0.001$ relative to the vehicle dressing. Both the 3% and 10% $TMBE_{Et}$ dressings were significant on day 7 relative to the standard silver dressing at $P < 0.05$. But while the 10% was again significant at $P < 0.05$ on day 13, the 3% dose was significant at $P < 0.001$ on day 13. Meanwhile, the 30% dose was not significant on either day 1, 3, 5, 7, or 13 against the standard dressed at $P > 0.05$ (See Fig 5.4c). All *Trichilia* groups were also found to be insignificantly different in the total ΔWd against the standard at $P > 0.05$. But significant for the 3%, significant for the 10% and 30% and significant for the standard silver dressing relative to the vehicle control group at $P < 0.05$, < 0.01, < 0.01 and < 0.001 respectively (See Fig 5.4d).

On the contrary, the petroleum ether extract of *Trichilia* ($TMBE_{Pt}$) had performed poorly as topical treatment for excised wounds. While the standard silver dressing showed a significant closure of wound diameter by day 13 as expected, wounds of the $TMBE_{Pt}$ treated groups were rather widening with increasing dose (Fig 5.4e & f). The Bonferroni's post-tests of two-way ANOVA pointed to an interesting development, in that, while the standard treated group showed a significantly positive wound closure ($P < 0.001$) on day 13, the 3% $TMBE_{Pt}$ treated group exhibited a significant wound widening ($P < 0.001$) on day 1 and significantly contracted ($P < 0.05$) on day 5. The situation was worse off in relation to wounds of the vehicle control animals relative to the vehicle control group (See Fig 5.4e & f).

Treatment response was slightly acceptable in the case of the 10% $TMBE_{Pt}$ group which showed a significant change in widening diameter on day 1 ($P < 0.05$) and a significant change in contracting diameter on day 13. The worsening change in wound diameter was then exacerbated in the 30% dose $TMBE_{Pt}$ dressing which manifested significant changes on day 1, 5, 7 and 13 ($P < 0.001$), but significant on day 3 at $P < 0.01$. The 3% $TMBE_{Pt}$ dressing had also significant differed from the standard dressing on day 1 and 3 ($P < 0.01$), and $P < 0.001$on day 7 and 5 and 13. While the 30% differed from the standard dressing on days 1, 3, 5, 7 and 13 at $P < 0.001$, the 10% dose significantly differed on days 3, 5, 7 and 13 at $P < 0.05$. Total ΔWd of all the $TMBE_{Pt}$ dressings were found to have significantly differed from the standard at $P < 0.001$ but only the 3% and standard dressing showed significant difference ($P < 0.01$) against the diseased with the 10% and 30% showing no significant difference ($P > 0.05$) relative to the vehicle control as shown in Fig 5.4e & f

Ethyl acetate as a solvent for the extraction of pharmacologically active ingredients from *Trichilia monadelpha* bark for the treatment of excised wound did not fare well (See Fig 5.4g & h). There was an increase mortality rate with increasing dose of treatment to the extent that the 30% $TMBE_{Ea}$ group had registered 100% mortality before the end of experimentation (Fig 4.10, G6). Corroborative of this fact, there were widening wound diameters in the 30% dose $TMBE_{Ea}$ dressing on day 13 under the Bonferroni's post-tests, which showed significant changes ($P < 0.01$). But the 3%, 10% and standard dressings were all not significantly different from the vehicle ($P > 0.05$).

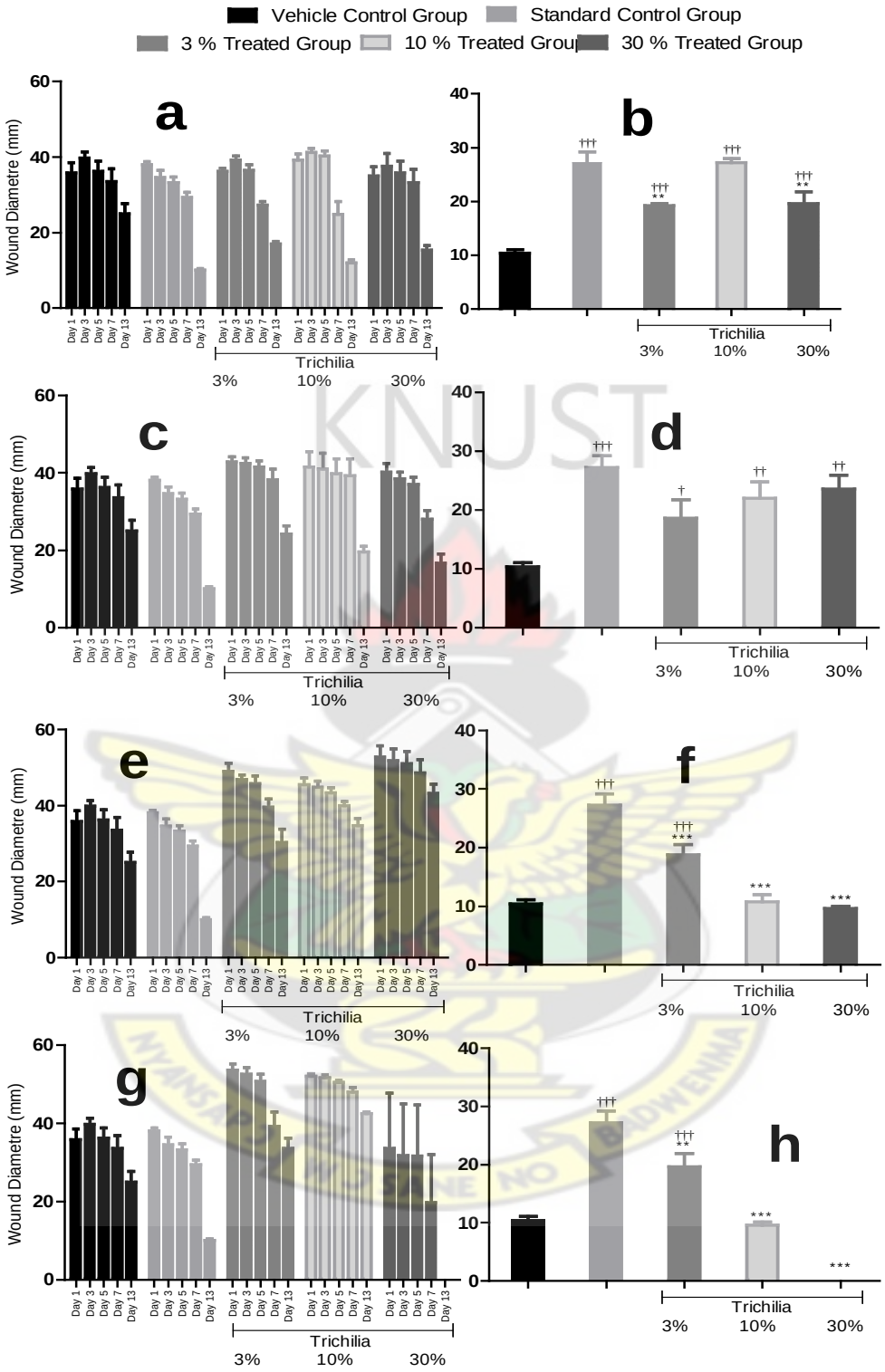


Figure 5.4: Effect of $TMBE_{Aq}$ on Change in Wound diameter (ΔWd). **(a & b)** $TMBE_{Aq}$ on ΔWd & Total ΔWd **(c & d)** $TMBE_{Et}$ on ΔWd & Total ΔWd **(e & f)** $TMBE_{Pt}$ on ΔWd & Total ΔWd **(g & h)** $TMBE_{Ea}$ on ΔWd & Total ΔWd. **Note:** (n=5, using Bonferroni's post-tests and comparing the mean ± S.E.M of each animal group's P.T

against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the vehicle control)

But, the 3% and 10% were significant different against the standard on day 13 but not the 30% dose (P < 0.05, < 0.001 and > 0.05) respectively Fig 5.4g. Total ΔWd showed significant difference for the standard, and 3% (P < 0.01), but insignificant for the 10% at P > 0.05. The 30% dose had however highly deteriorated against the vehicle as in Fig 5.4h. Relative to the standard dressing, the 10% and 30% were significant at P < 0.001, while the 3% was significantly different from the standard at P < 0.01 (See Fig 5.4h).

5.3.2 Effect of TMBE on Wound contraction (Wc)

Wound contraction was a tale in the $TMBE_{Aq}$ dressings. All but the standard control had progressive acute inflammation with widening wounds on day 1 as shown in Fig 5.5a & b. However, the acute inflammatory situation was not stayed and so by day 3 post-wounding, wound contraction was drastic and consistent up to day 13 in almost all the animal groups especially for the 10% $TMBE_{Aq}$ group but which lost its consistency to a seeming antagonistic component of the extract. There was significant contractions showing on day 3 and significant on day 13 for the standard group (P < 0.05 and < 0.001), relative to the vehicle. While the 3% and 10% $TMBE_{Aq}$ dressings showed significant contraction differences on day 7 (P < 0.05 and < 0.001), the 30% $TMBE_{Aq}$ had a significant (P < 0.01) on only day 13. The 3% $TMBE_{Aq}$ was also significant on day 3 and 13 relative to the standard (P < 0.05 and < 0.01), while the 10% was significant on day 7 and 13 (P < 0.001 and < .05) with the 30% registering no significant difference against the standard (See Fig 5.5a). Average Wc was significantly different for the 3% and 30% against the vehicle (P < 0.01), while the standard and 10% treatments were found to be significant at P < 0.001. None of the $TMBE_{Aq}$ groups showed significant difference

against the standard (Fig 5.5b). Wounds of animals among these groups were generally incomplete by the end of experimentation as shown in Fig 5.7 – 5.9 & 5.10.

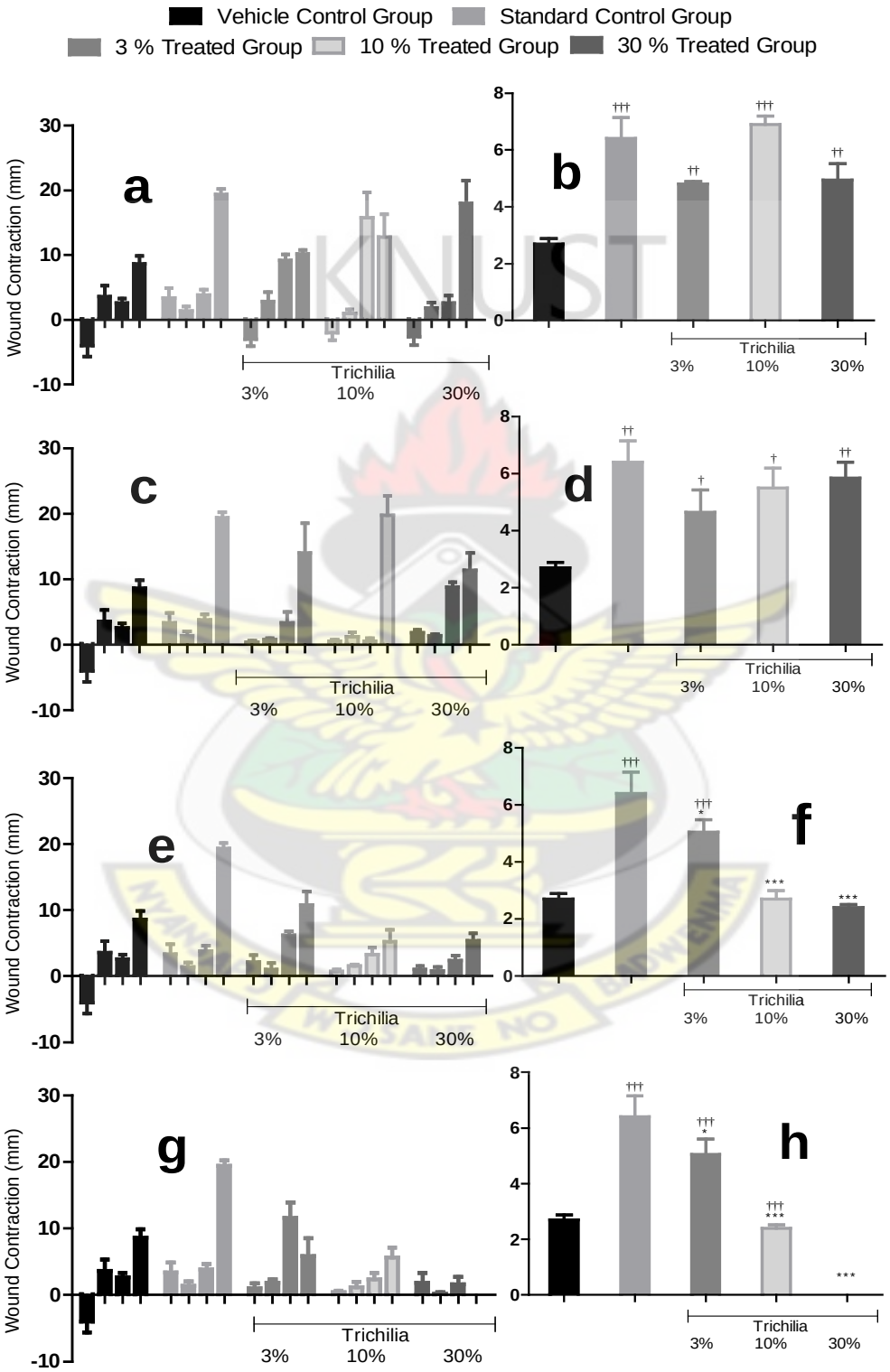


Figure 5.5: Effect of $TMBE_{Aq}$ on Wound contraction (Wc). **(a & b)** $TMBE_{Aq}$ on Wc, **(c & d)** $TMBE_{Et}$ on Wc, **(e & f)** $TMBE_{Pt}$ on Wc, **(g & h)** $TMBE_{Ea}$ on Wc. **Note:** (n=5, using Newman-Keuls test and comparing the mean ± S.E.M of each animal group against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the vehicle control)

As an effective topical medication for excised/surface wounds $TMBE_{Et}$ had effectively halted acute inflammation and registered some marginal wound contraction on day 1 post wounding in all three doses. The rate of contraction was consistent as the days passed and dose dependent (Fig 5.5c). The rate of contraction was however maximal on day 13 of the 10% *Trichilia* treated group. But while there were significant contractions on day 3 and 13 ($P < 0.05$ and < 0.001) of the standard dressing, there was also a significant contraction on day 13 of the 10% $TMBE_{Et}$ dressed ($P < 0.001$). Contraction was significant on day 7 of the 30% dose ($P < 0.05$) and not significant at all on the 3% dressed ($P > 0.05$) in relation to the diseased. All $TMBE_{Et}$ groups were also found to have insignificantly contracted relative to the standard dressing (Fig 5.5c). Meanwhile, average Wc showed some significance for the 3% and 10% at $P < 0.05$ and $P < 0.01$for the 30% and standard groups, with no significant difference among the *Trichilia* treatments ($P > 0.05$) against the standard (Fig 5.5d).

On the other hand, wound contraction for the $TMBE_{Pt}$ was as poor. While the standard silver dressing showed a significant contraction rate by d 13 as expected, wounds of the $TMBE_{Pt}$ dressings though consistent, proceeded at a slow pace, widening with increasing dose as in Fig 5.5e & f. Wound contraction for the standard group of petroleum ether ($TMBE_{Pt}$), was significant on day 3 and 13 at $P < 0.001$ and on only day 3 for the 3% $TMBE_{Pt}$ at $P < 0.001$. Then significant on same day for the 10% and 30% $TMBE_{Pt}$ groups at $P < 0.001$, < 0.05 and < 0.01 respectively. Interestingly, all the $TMBE_{Pt}$ dressings had registered significant difference against the standard at $P < 0.01$. Average Wc was also significantly different for the 10% and 30% $TMBE_{Pt}$ as against the standard at $P < 0.001$ and $P < 0.05$ for the 3%. Meanwhile, the 3%

and the standard were found to have significant difference against the vehicle at $P < 0.001$ with the 10% and 30% showing no significant difference as shown in Fig 5.5e.

Corroboratively, there was failed wound contraction in the $TMBE_{Ea}$ dressings, widening among the 10% and 30% dose $TMBE_{Ea}$ groups (See Fig 5.5g & h). The standard dressing significantly contracted on day 3 and 13 ($P < 0.001$) against the vehicle dressing of $TMBE_{Ea}$. The 3% significantly contracted on day 3 and on day 7 ($P < 0.05$ and < 0.001). The 10% had no significant difference ($P > 0.05$) while the deteriorating 30% $TMBE_{Ea}$ dressing significantly differed against the vehicle on day 3 and 13 ($P < 0.01$ and <0.001). In comparison with the standard dressing, the 3% again registered significant difference on day 7 and 13 ($P < 0.001$) and not significant for the 10% and 30% on day 13 as in Fig 5.5g. On the average, the standard, 3% and 10% dressings all had contracted wounds ($P < 0.001$), relative to the vehicle dressing, with the 30% dose highly deteriorating relative to the vehicle dressing. Same was the case for the 3% and 10% which had significant difference against the standard ($P < 0.05$ and < 0.001) as depicted in Fig 5.5h.

5.3.3 Effect of TMBE on Wound size (Ws)

Reduction in wound size was appreciable among the 10% $TMBE_{Aq}$ dressed rats comparable to the 30% dose $TMBE_{Aq}$ rats. The promotion of excision wound closure is much clearer on Fig 5.6a & b, pointing to an amazingly good size comparable to other *Trichilia* dressings. The wounds sizes varied insignificantly ($P > 0.05$) in all treatment groups but for the standard dressed which showed some significant difference ($P < 0.05$) against the vehicle control. Wound sizes among the $TMBE_{Et}$ dressings were much good. This is apparently clearer on Fig 5.6c & d. The $TMBE_{Et}$ dressed wounds had also reduced insignificantly ($P > 0.05$) in all treatments against the vehicle. The 3% and 10% $TMBE_{Et}$ had however significantly ($P < 0.05$)

reduced in sizes relative to the standard silver dressing but not the 30%. On average Ws, there was no significant change among all animal groups as in Fig 5.6d.

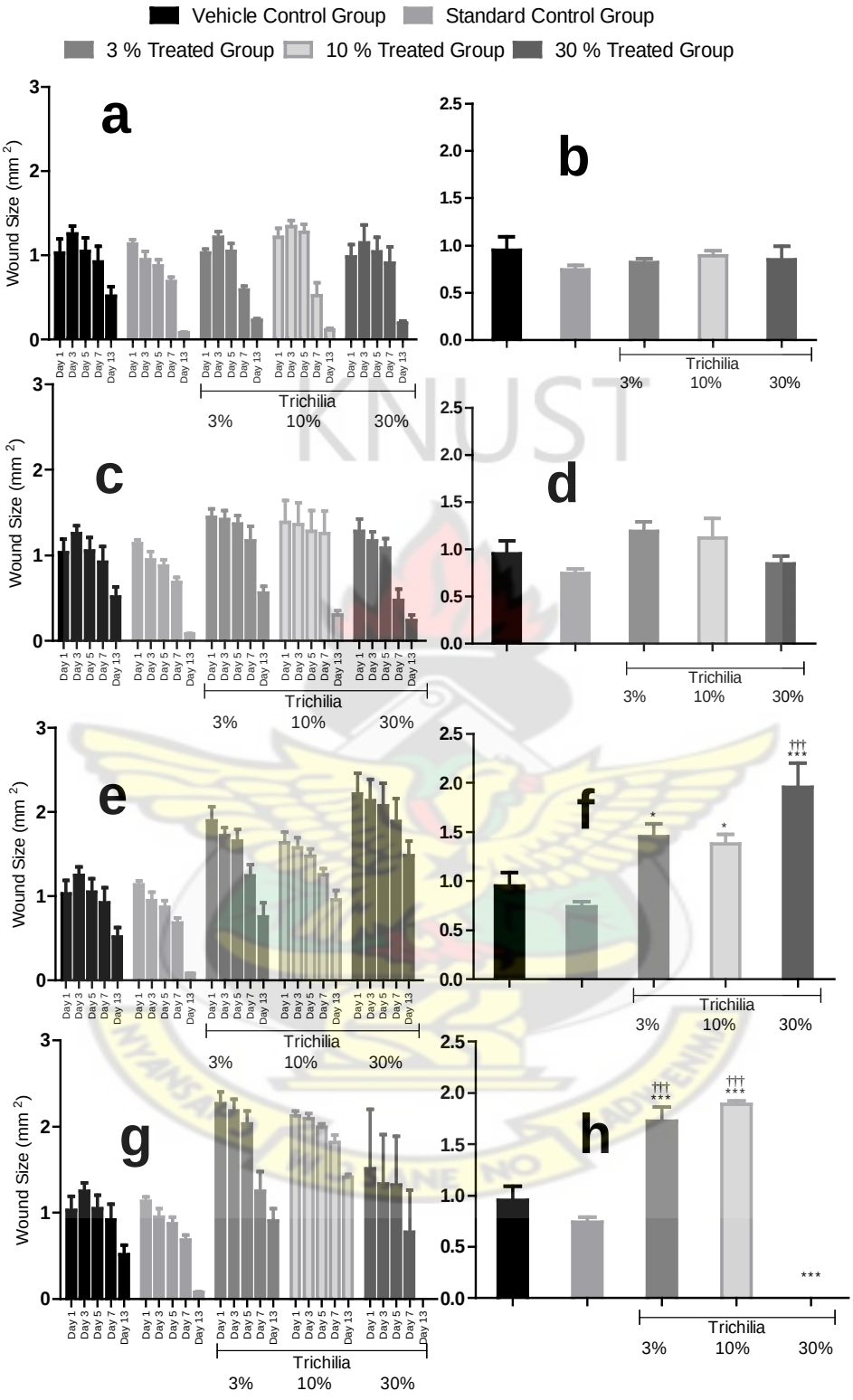


Figure 5.6: Effect of $TMBE_{Aq}$ on Wound size (Ws). **(a & b)** $TMBE_{Aq}$ on Ws, **(c & d)** $TMBE_{Et}$ on Ws, **(e & f)** $TMBE_{Pt}$ on Ws, **(g & h)** $TMBE_{Ea}$ on Ws. **Note:** (n=5, using Bonferroni's post-tests and comparing the mean ±

S.E.M of each animal group's P.T against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the vehicle control)

The worsening in wound size of the $TMBE_{Pt}$ dressing was exacerbated in the 30% dose which manifested minimal wound contraction on d 1, 5, 7 and 13 (See Fig 5.6e). Thus, wound sizes were larger among the $TMBE_{Pt}$ groups in a dose dependent fashion as in Fig 5.16e & f. Wound sizes of $TMBE_{Pt}$ were significantly larger on day 1 and 5 ($P < 0.001$ and $P < 0.05$) for the 3% $TMBE_{Pt}$, but not for the 10% and standard ($P > 0.05$). The 30% dressing was however significantly larger relative to the vehicle ($P < 0.001$). But there were no significant differences in wound sizes of the standard, 3% and 10% $TMBE_{Pt}$ compared to average Ws of the vehicle ($P > 0.05$), but for the 30% dressing ($P < 0.001$). Meanwhile, the 3% and 10% dose dressings had significantly increased in sizes relative to wounds of the standard ($P < 0.05$) and the 30% dose dressing was much more large ($P < 0.001$) relative to the standard (See Fig 5.6f).

The $TMBE_{Ea}$ failed to facilitate wound healing which was clearer in the determination of wound size (Fig 5.6g & h). There was an increase in wound size with increasing dose, the largest of all occurring among animals of the 30% dose $TMBE_{Ea}$ (See Fig 5.6h). The results showed no significant difference in wound size of the standard and 30% $TMBE_{Ea}$ relative to the vehicle ($P > 0.05$), but for the 3% on day 1 and 5 ($P < 0.01$ and $P < 0.05$). Relative to wound sizes of the $TMBE_{Ea}$ creams to those of the silver cream, the 3% was significantly larger on day 1($P < 0.05$) and on day 3 and 5 ($P < 0.01$), with wounds of the 10% significantly increasing in sizes on days 1, 3, 5, 7 and 13 ($P < 0.001$). Wounds of the 30% were not any larger ($P > 0.05$) than those of the standard (Fig 5.6g). Averagely, the standard dressing was not significantly ($P > 0.05$) different from the vehicle but both the 3% and 10% had significantly larger relative to the vehicle ($P < 0.001$). However, the 30% had significance increment in wound sizes relative

to both the vehicle and standard ($P < 0.001$), while the 3% and 10% were only larger in comparison to the standard ($P < 0.001$) as shown in Fig 5.6h.

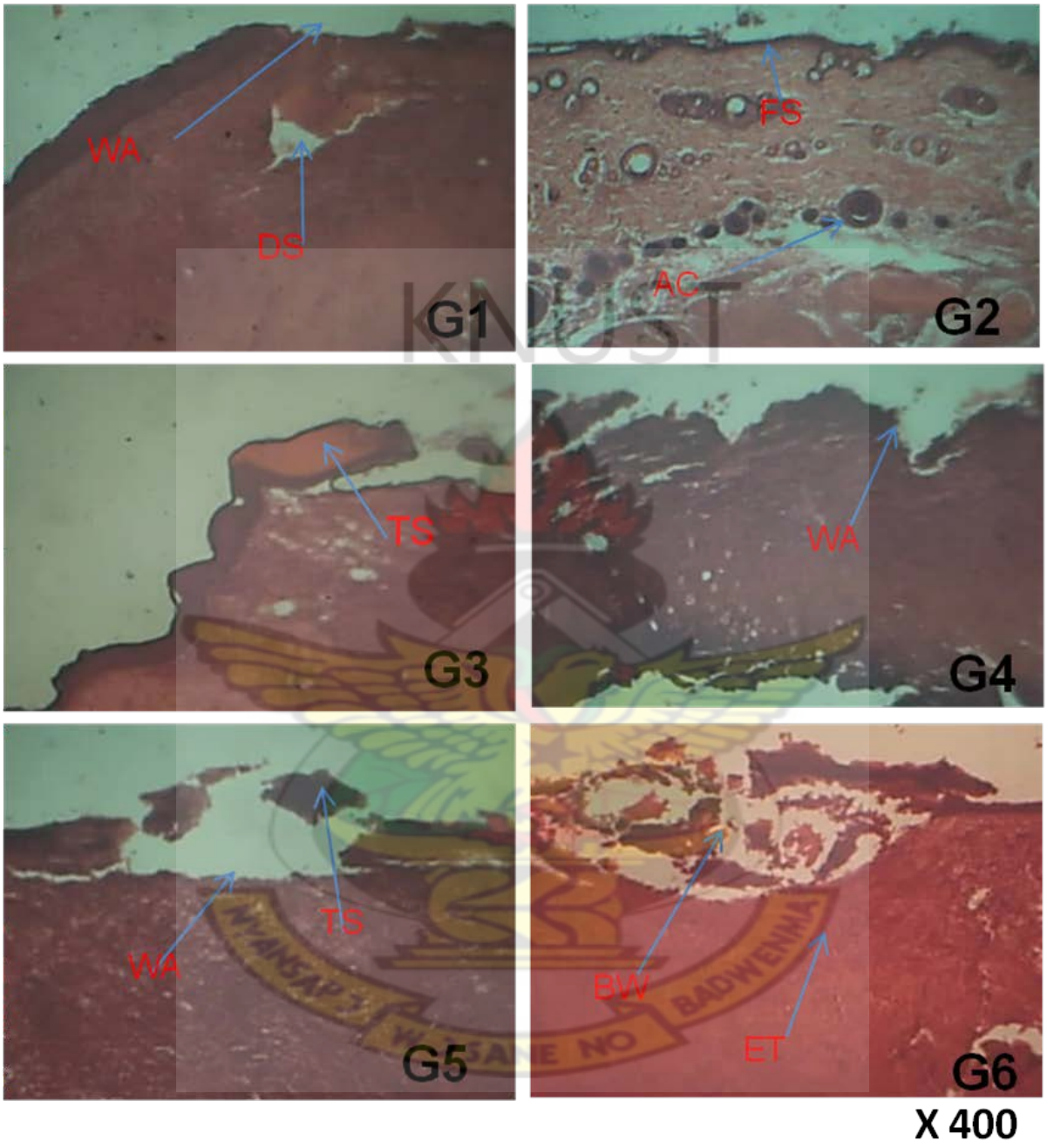


Figure 5.7: The Effect of 3% TMBE on Excision Wounds; (G1) = Disease Control Group; incomplete wound with dead-space and erythema, (G2) = Standard Control Group; complete healing with formed scar and marked apoptosis, (G3) = $TMBE_{Aq}$; absences of sepsis with rapid contraction (G4) = $TMBE_{Et}$; absences of sepsis with improved wound contraction (G5) = $TMBE_{Pt}$; poor wound healing with inconsistent scaring (G6) = $TMBE_{Ac}$; persistent inflammation with sloughing and bleeding. **Note:** AC; Apoptotic cells, BW; Bleeding wound, DS; Dead space, ET; Erythematous tissue, FS; Formed scar, SW; Sloughing wound, TS; Torn scar, WA; Wound area.

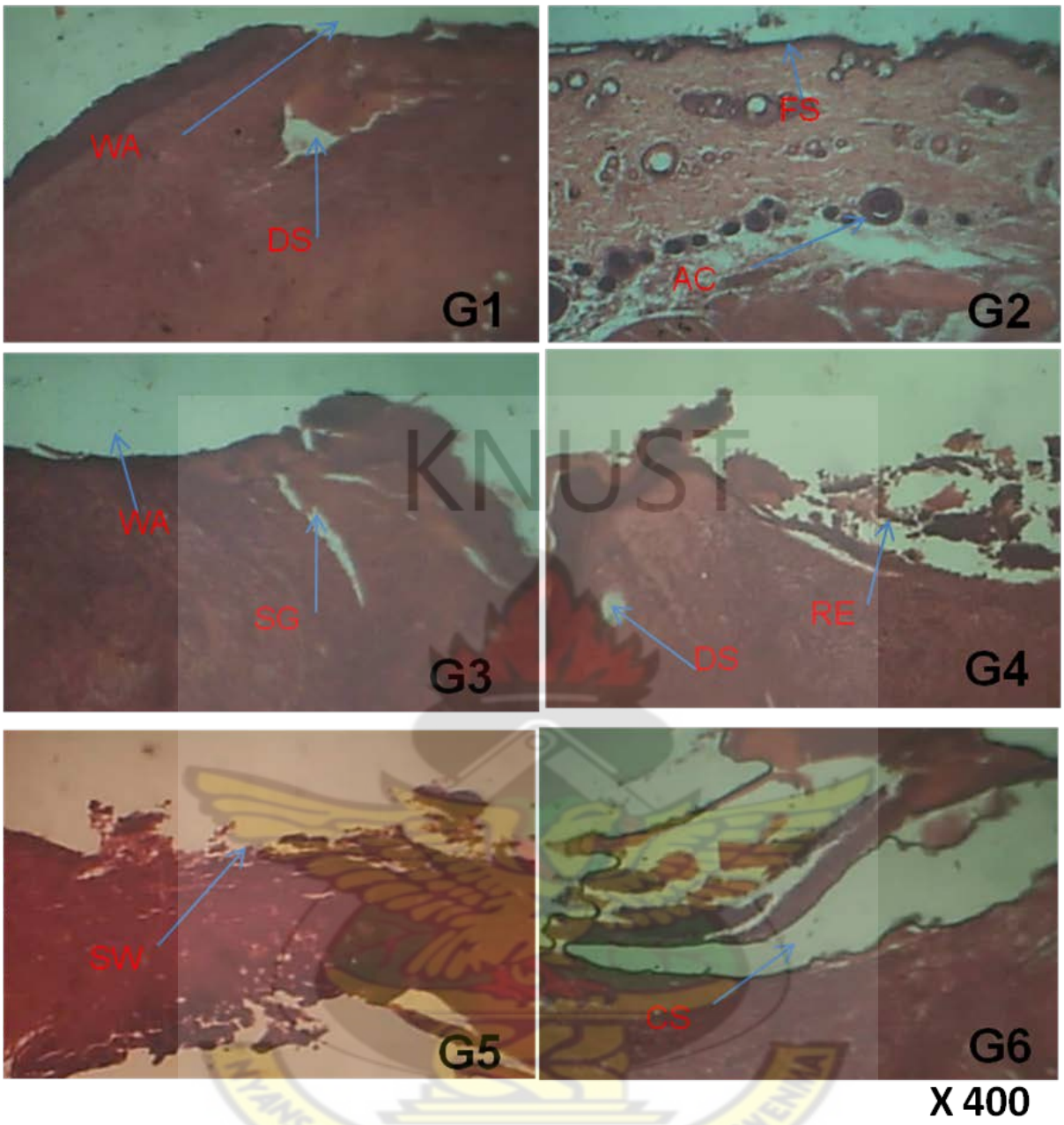


Figure 5.8: The Effect of 10% TMBE on Excision Wounds; (G1) = Disease Control Group; incomplete wound with dead-space and erythema, (G2) = Standard Control Group; complete healing with formed scar and marked apoptosis (G3) = $TMBE_{Aq}$; appreciable wound healing with little or no inflammation (G4) = $TMBE_{Et}$; appreciable wound healing with re-epithelialisation (G5) = 300 mg $TMBE_{Pt}$; failed wound healing with sloughing of cutaneous tissue (G6) = $TMBE_{Ac}$; failed wound healing with sepsis. **Note:** AC; Apoptotic cells, CS; Chronic sore, DS; Dead space, FS; Formed scar, RE; Re-epithelialisation, SG; Stretch gap, SW; Sloughing wound, WA; Wound area.

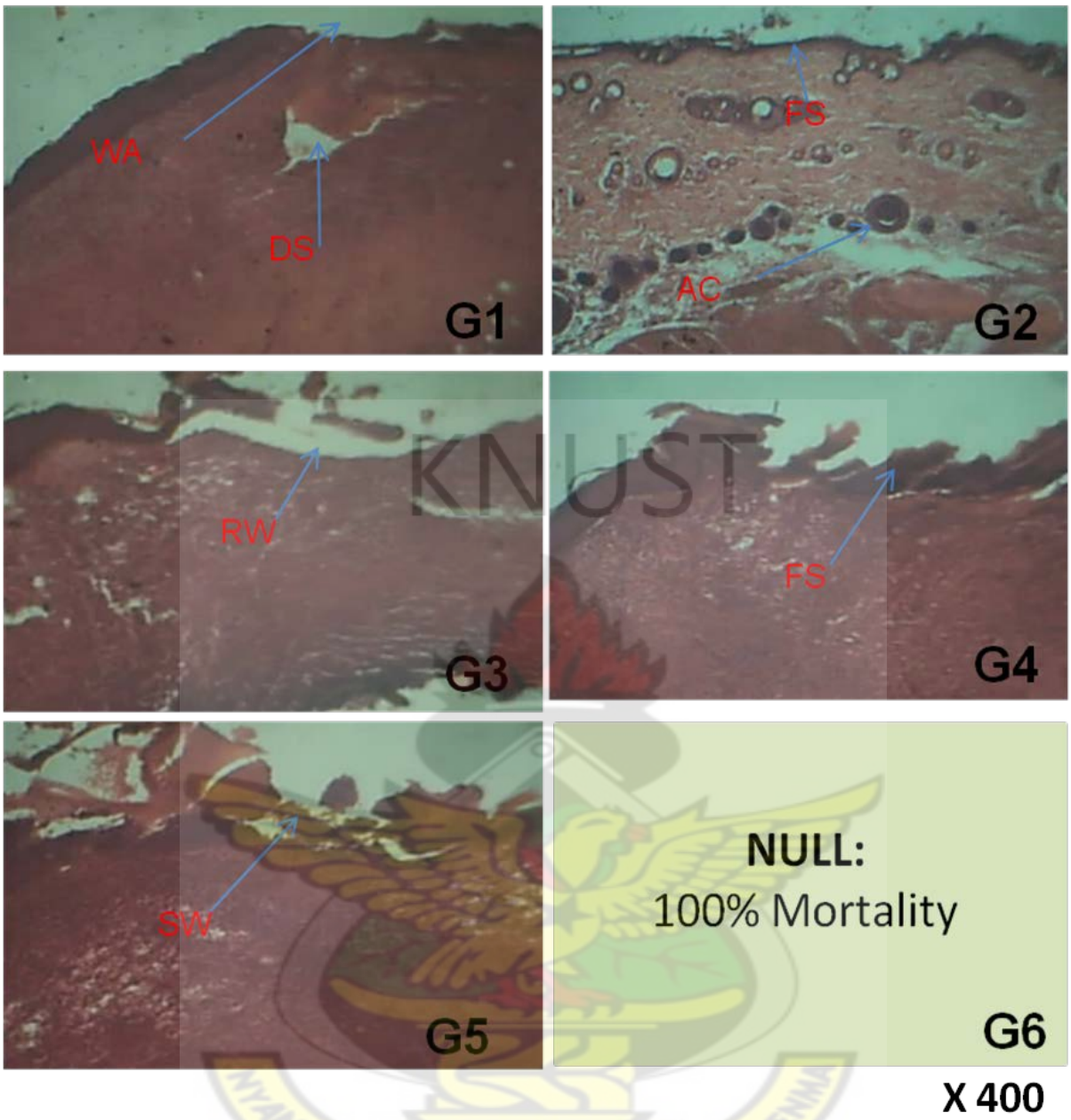


Figure 5.9: The Effect of 30% TMBE on Excision Wounds; (G1) = Disease Control Group; incomplete wound with dead-space and erythema, (G2) = Standard Control Group; complete healing with formed scar and marked apoptosis (G3) = $TMBE_{Aq}$; slowed healing pointing to seeming antagonism, (G4) = $TMBE_{Et}$; marked wound healing wither-epithelialisation and well formed scar, (G5) = 300 mg $TMBE_{Pt}$; stasis wound with re-epithelialisation and pus underneath (G6) = $TMBE_{Ac}$; chronic wound with 30% fatality. **Note:** AC; Apoptotic cells, DS; Dead space, FS; Formed scar, RW; Remaining wound, SW; Sloughing wound, WA; Wound area.

5.3.4 Effect of TMBE on Rate of Wound healing (Rwh)

Rate of healing was also was consistent among the $TMBE_{Aq}$ dressings as the days pass and dose dependent as in Fig 5.10a & b. Rate of healing in the $TMBE_{Aq}$ was only significantly different on day 3 ($P < 0.05$) and on day 7 for the standard but $P < 0.01$ for 3% dressing and $P < 0.001$for the 10% dressing. The 30% $TMBE_{Aq}$ group showed no significant ($P > 0.05$) rate of healing on either day 3, 5, 7 or 13. The 3% dose was subsequently found to be significant ($P < 0.05$) against the standard and $P < 0.001$ for the 10%. The 30% was not at all significant in relation to the standard (Fig 5.20a). Average rate of healing was significant for the standard and 3% dressings at $P < 0.01$ and $P < 0.001$ for the 10% but not significant as against the standard. Meanwhile, only the 10% and 30% groups were significant ($P < 0.05$) against the standard silver dressing as shown in Fig 5.10b.

The average rate of healing peaked among the 30% $TMBE_{Et}$ group compared to the standard control (Fig 5.10c & d). At the final phase, the wounds underwent rapid contraction resulting in a smaller amount of apparent scar tissue (See Fig 5.11, G4). In much the same way, there was no significant ($P > 0.05$) rate of healing on day 13 for both the 3% and 30% $TMBE_{Et}$ but the 30% dose had significant rates on day 3 and 7 ($P < 0.01$ and < 0.001). However, the 10% $TMBE_{Et}$ though slow in healing, had significant rate ($P < 0.05$) on day 13, closely following the standard, which showed significant rates of healing on day 3 and 13 ($P < 0.001$ and < 0.01) relative to the vehicle. The 10% and 30% $TMBE_{Et}$ but not the 3% were also significant against the standard ($P < 0.001$, 0.001 and > 0.05) as in Fig 5.10c. Averagely Rwh showed significant rates for the standard and 30% dressings against the vehicle ($P < 0.001$) with no significant difference for the 3% and 10% $TMBE_{Et}$ dressings ($P > 0.05$). The rate had however differed ($P < 0.001$) for the 3% and 10% as against the standard but not the 30% dressed (See Fig 5.10d).

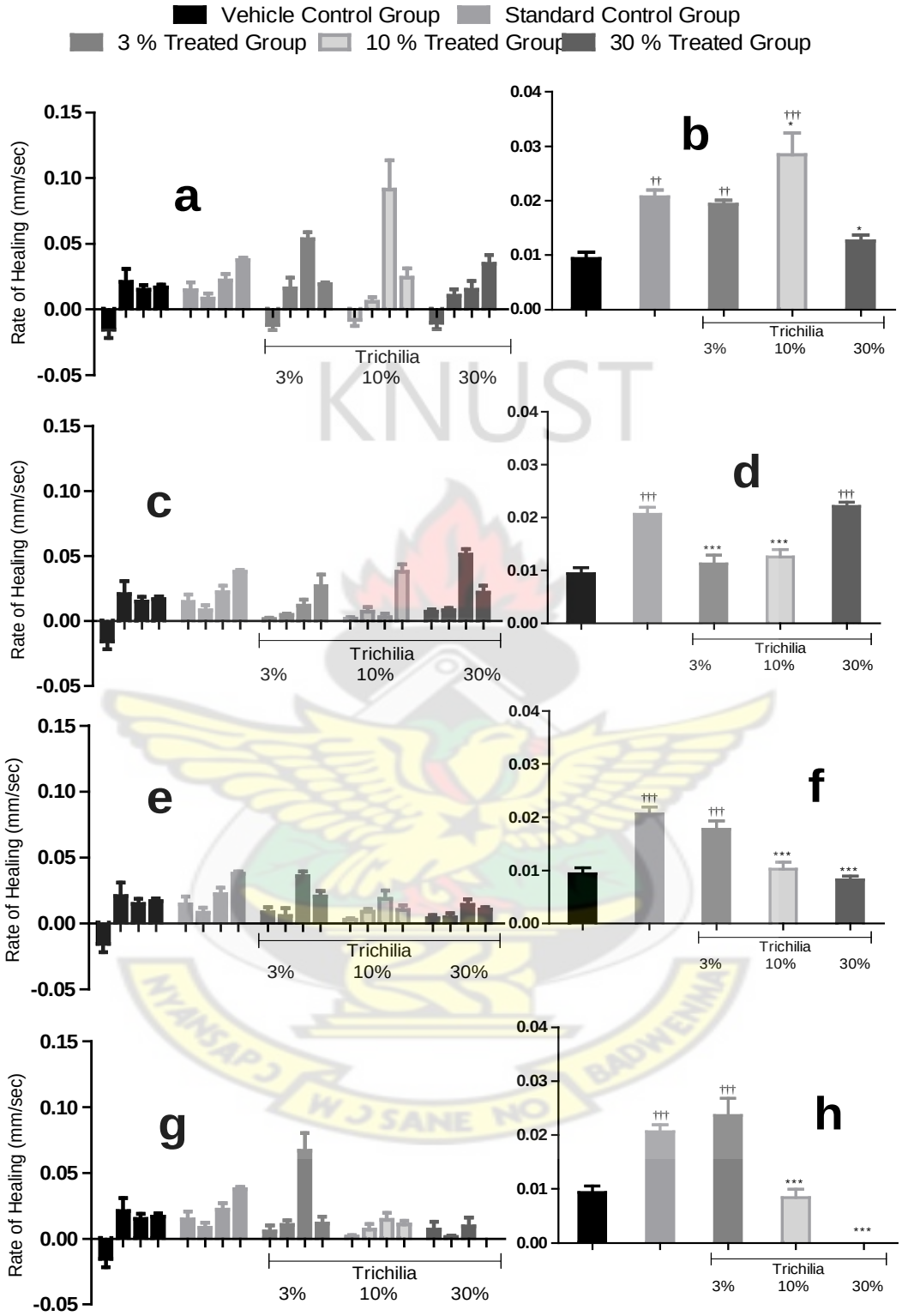


Figure 5.10: Effect of $TMBE_{Aq}$ Rate of Wound healing (Rwh). **(a & b)** $TMBE_{Aq}$ on Rwh, **(c & d)** $TMBE_{Et}$ on Rwh, **(e & f)** $TMBE_{Pt}$ on Rwh, **(g & h)** $TMBE_{Ea}$ on Rwh. **Note:** (n=5, using Newman-Keuls test and comparing the mean ± S.E.M of each animal group against the normal control, †††$P < 0.001$; ††$P < 0.01$, †$P < 0.05$, and ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$ against the vehicle control)

The rate of wound healing for $TMBE_{Pt}$ creams reflected the results obtained for wound contraction and wound size above (Fig 5.11, G5). The rate of healing was poor among the $TMBE_{Pt}$ dressings (See Fig 5.10e & f).The rate of healing in the $TMBE_{Pt}$ was about same for the standard control ($P < 0.001$ and <0.01) for days 3 and 13. The 3% group had significant rates of healing on day 3 and 7 ($P < 0.01$) but $P < 0.05$ on day 3 for the 10% group. The 30% $TMBE_{Pt}$ had a significant rate of healing ($P < 0.05$) on day 3 and 5 but not 7 and 13. The 10% and 30% were also found to have significantly healed on day 13 relative to the standard ($P < 0.001$) as in See Fig 5.10e. The average rate of healing saw the 10% and 30% significantly differing from the standard ($P < 0.001$) but not for the 3%. Meanwhile, the standard and 3% had significantly healed relative to the vehicle ($P < 0.001$) and the 10% and 30% had no significant difference ($P > 0.05$) against the vehicle as in Fig 5.10f. However, the rate of healing was slowest among the $TMBE_{Ea}$ dressings with the 10% and 30% dose groups showing little or no healing (Fig 5.10g & h). Rate of wound healing in the $TMBE_{Ea}$ was significant for the standard silver dressing on day 3 and 13 against the vehicle ($P < 0.001$) and while the 3% had significant rate of healing on day 1 and significant on day 7 ($P < 0.05$ and < 0.001), the 30% was only significant on day 3 ($P < 0.05$) and the 10% not significant on either days 1, 3, 5, 7or 13 as in Fig 5.10g. Relative to the standard, the 3% was significant ($P < 0.001$) on day 7 and on day 13 ($P < 0.01$), with the 10% and 30% showing significant rate of healing on day 13 against the standard silver dressing ($P < 0.01$ and <0.001). Average rate of healing showed significant rate of healing for the standard ($P < 0.001$) and 3% $TMBE_{Ea}$ cream against the vehicle. The 10% dose was not significantly different ($P > 0.05$) and the 30% had significant slow rate of healing relative to the vehicle dressing. Meanwhile, both the 10% and 30% $TMBE_{Ea}$ dressings had significantly healed ($P < 0.001$) relative to the standard but not the 3% dose as shown in Fig 5.10h.

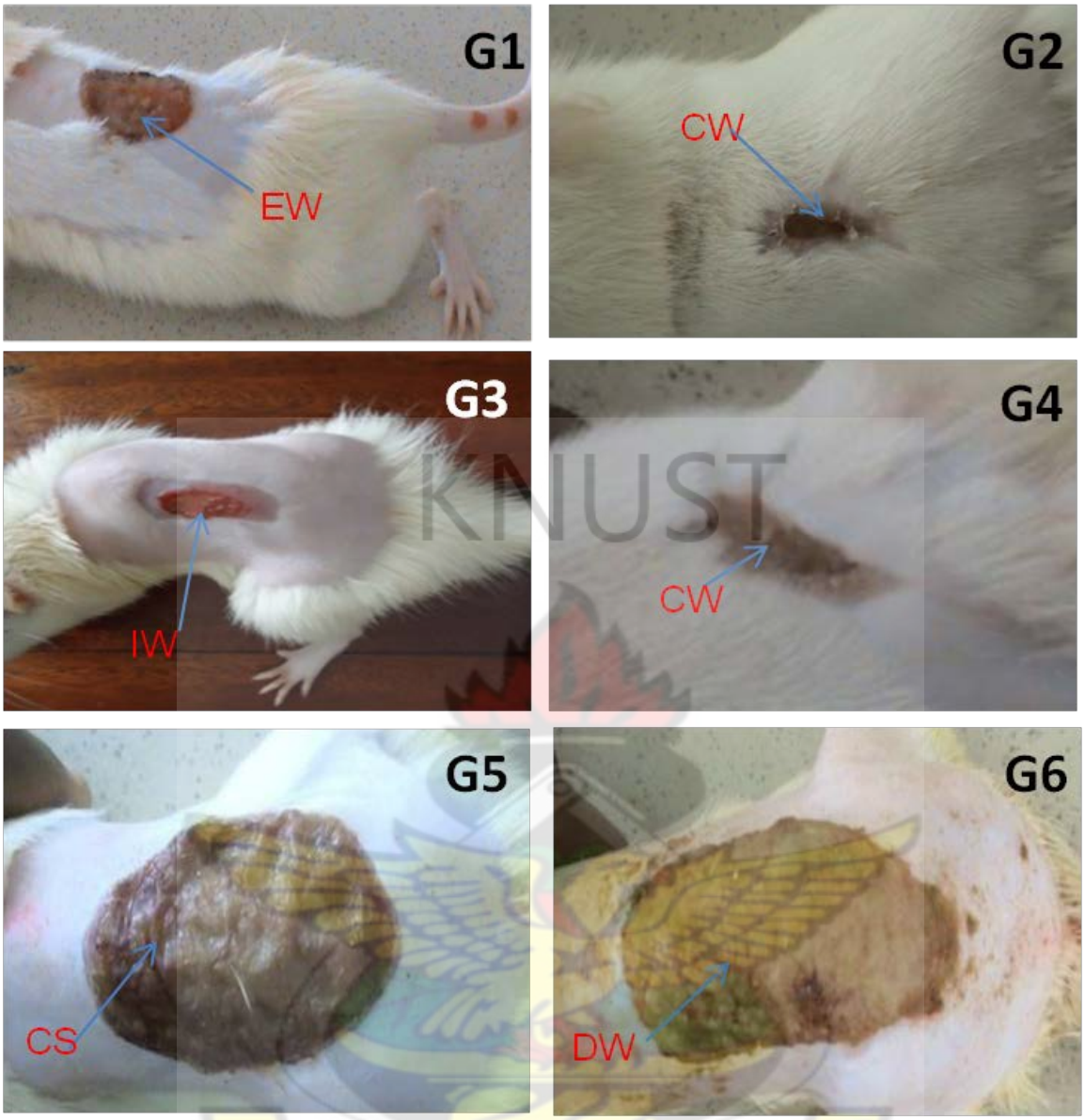


Figure 5.11: The Effect of TMBE on Excision Wounds; (G1) = Disease Control Group; incomplete wound with dead-space and erythema (G2) = Standard Control Group; complete healing with formed scar and marked apoptosis (G3) = $TMBE_{Aq}$; incompletely healed wound with re-epithelialisation pointing to slow healing (G4) = $TMBE_{Et}$; completely healed wound with re-epithelialisation and well formed scar (G5) = 300 mg $TMBE_{Pt}$; stasis wound with cellular activity underneath, (G6) = $TMBE_{Ea}$; chronic wound with pus underneath, evident of secondary infection. **Note:** CS; Chronic sore, CW; Complete wound, DW; Defatted wound, EW; Erythematous wound, IW; Incomplete wound.

5.4 DISCUSSION

Because wounding is severe in the excision wound model, it allowed for the investigation of central tissue movements associated with repair, starting with haemorrhage followed by re-epithelialisation, granulation tissue formation, and angiogenesis (Paddock *et al.*, 2003). Generally the anti-oxidant property of *Trichilia* bark extracts as demonstrated in the rapid screening assay had offset wound stressing factors in the topical treatment. Wounds of both vehicle control and aqueous extract ($TMBE_{Aq)}$ dressings had enlarged on day 1 post wounding, an indication of progressing pathology due to acute inflammation. By day 3, the acute inflammatory situation had receded. All other groups including the standard control group did not exhibit an increase in wound width on day 1, an indication of effective drug intervention against acute inflammatory responses. It was observed that, a few hours after wounding, the wounded area had soon been closed by a thin scab, which became hardened within the first 2 days of repair in accordance with Paddock's accession (2003). Wound size had generally reduced by the end of experimentation relative to their original sizes. Wound contractions in the petroleum ether and ethyl acetate groups were however so insignificant that one could safely attribute the marginal reduction in sizes to forced contraction.

Trichilia bark extracted with ethanol ($TMBE_{Et}$) as an organic solvent, had a relatively higher activity on excision wound (ExW) and could be a preferred treatment. Albeit, wounds were generally wider among the ethanolic extract treated groups from onset. Reduction in wound diameter was slow, constant and in a dose dependent fashion relative to the standard silverzine dressing. On the contrary, the petroleum ether extract of *Trichilia* ($TMBE_{Pt}$) had performed poorly as topical treatment for excised wounds. While the standard silverzine dressing showed a significant closure of wound diameter by day 13 as expected, wounds of the petroleum ether extract treated groups were rather widening with increasing dose.

The situation was worse off in relation to wounds of the vehicle control animals. The petroleum ether treatment had however proven to be anti-inflammatory, and had halted the acute inflammatory phase, characterized by epithelialisation and angiogenesis (Nayak and Pereira, 2006) relative to the vehicle control group. Therefore this failed treatment could be attributable to local and/or systemic toxicity leading to impaired healing.

Though proven to be an anti-oxidant and anti-inflammatory, the ethyl acetate extract of *Trichilia monadelpha* bark ($TMBE_{Ea}$) for the treatment of excised wound was a failed attempt as it did not proof scientifically to be worth the course. There was an increase mortality rate with increasing dose of treatment to the extent that the 30% $TMBE_{Ea}$ group had recorded 100% mortality before the end of experimentation. Corroborative of this fact, there were widening wound diameters in the 10% and 30% dose $TMBE_{Ea}$ groups with evidence of persistent defatting on the wound surface.

The average rate of healing peaked among the 30% dose $TMBE_{Et}$ dressed group comparable to the standard control group. The $TMBE_{Et}$ must have demonstrated a significant increase in the hydroxyproline content of the granulation tissue indicating increased collagen turnover. Collagen, the major component which strengthens and supports extra cellular tissue is composed of the amino acid and hydroxyproline, which has been used as a biochemical marker for tissue collagen (Nayak and Pereira, 2006). The wound healing property of $TMBE_{Et}$, a catholic solvent, may be attributed to the phytoconstituents present in the plant, and the hastened process of wound healing could be a function of either the individual or the additive effects of the phyto-constituents.

Recent studies have shown that phytochemical constituents like flavonoids and triterpenoids are known to promote the wound healing process mainly due to their astringent and antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelialisation (Nayak and Pereira, 2006). At the final phase, the wounds underwent rapid contraction resulting in a smaller amount of apparent scar tissue.

Evident of the in-effectiveness of the Petroleum ether extract of *Trichilia* ($TMBE_{Pt}$), reflected the results obtained for wound contraction and wound size as above and also failed to reduce the scar size. The rate of healing was poor among the $TMBE_{Pt}$ dressings, however, the rate of healing was slowest among the $TMBE_{Ea}$ dressed groups with the 10% and 30% dose treated groups showing little or no healing. To this extend, one could confidently associate this development to some local and/or systemic toxicity, especially when all $TMBE_{Ea}$ dressed wounds showed marked evidence of defatting. In the early phases of wound healing, chemokines and cytokines regulate chemotaxis and activation of inflammatory cells, as well as synthesis of proteases and protease inhibitors but $TMBE_{Ea}$ could not sustain this process.

Comparatively, the results clearly indicated that, the aqueous and ethanolic extracts had halted inflammation and subsequently facilitated healing, but not the petroleum ether and ethyl acetate extracts. Most wounded animals were able to cope well with the injuries (Paddock *et al.*,2003).

5.5 CONCLUSION

By and large, the aqueous extract was found to have facilitated excision wound healing but not as much as did the ethanolic extract. The petroleum ether and ethyl acetate extracts were however poor topical wound treatment alternatives.

CHAPTER SIX

6.0 GENERAL DISCUSSION

General Findings, Inferences and Implications

6.1 General Overview

In the indomethacin induced UC, ethanolic extract of *Trichilia* revealed persistent mucosal ulceration in the disease control and 30 mg/kg but not the 100 mg/kg and 300 mg/kg dose treated groups, and its activity was much similar in the acetic acid Induced UC. Meanwhile, the aqueous extract could significantly restore mucosal integrity in the 100 mg/kg and 300 mg/kg treated groups for the acetic acid induced UC, relative to the ulcerated and oedematous mucosa of 30 mg/kg group, pointing to a marked response to treatment in dose dependent fashion. And while the petroleum ether extract manifested crypt abscesses and globular mucosae in the 100 mg/kg and 300 mg/kg treated groups of acetic acid induced colitic rats, the ethyl acetate extract revealed serrated, cryptic and oedematous mucosae of same among the 100 mg/kg and 300 mg/kg dose treated groups, evident of persistent disease.

Corroboratory to earlier claims in the Ghana Herbal Pharmacopoeia (2007), investigation of the anti-microbial role of *Trichilia monadelpha* on intestinal microflora in colitic rats suggested a dose dependent activity. There was a 25%, 11% and 7% microbial infestation among animals of the 30 mg/kg, 100 mg/kg and 300 mg/kg *Trichilia* treated groups relative to a 32% occurrence in disease control. Therefore, *Trichilia* was found to be sensitive to colonic microflora. As such, if microflora is beneficial to the host, then much could be lost in the form of micronutrient supply and protection against opportunistic pathogens.

On the excision wounds, the aqueous extract showed appreciable rate of healing in the 3% and 10% dressed groups but not in the 30% dose dressing with incomplete healing by day 13 of treatment. In the ethanolic extract on the other hand, rate of wound healing was dose dependent and comparable to the standard silver dressing.

Most wounds had completely healed by day 13, with evidence of epithelialisation scar. But the petroleum ether extract showed a relatively poor rate of wound healing, evident with sepsis leading to chronic wounds, and the ethyl acetate extract was the poorest, characteristic of ineffective treatment, evident by increased mortality peaking to 100% in the 30% dressed group.

CHAPTER SEVEN

7.0 CONCLUSION AND RECOMMENDATIONS

General Conclusions and Recommendations

7.1 CONCLUSION

To this extend, it is established that; the aqueous extract proved to be a strong anti-oxidant and was highly effective in the treatment of UC but slightly effective in the healing of excised wounds. The ethanolic extract had appreciable anti-oxidant property and was marginally effective in the treatment of UC but highly effective in the healing of excised wounds. The petroleum ether and ethyl acetate extracts did not proof to be effective treatment options in both cases. *Trichilia* was also found to be detrimental to colonic microflora and could be effective against infectious colitis.

7.2 RECOMMENDATIONS

It is worth mentioning that, since the aqueous and ethanolic extracts of *Trichilia monadelpha* bark were effective on ulcerative colitis and excision wounds but detrimental to colonic microflora, further exploits ought to be considered on the antimicrobial role using modern molecular methods to reliably identify the isolates. Especially when characterization sufficient to identify individual isolates often require hundreds of hours to conduct as much as 20 biochemical tests, interpretation of which will challenge even the most experienced microbiologist.

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