CHAPTER ONE

INTRODUCTION

1.1 Background

Medicinal plants have continued to attract attention in the global search for effective methods of using plants' parts for the treatment of many diseases affecting humans (Alexandra *et al.,* 2018). Many important drugs used in medicine today are directly or indirectly derived from plants due to their bioactive constituents such as; alkaloids, steroids and tannins (Thomford *et al*., 2018)**.** Peptic ulcers are sores in the lining of the stomach or small intestine. They occur when the protective factors of the gastro-intestinal tract are overwhelmed by the "aggressive factors" (https://www.medicalnewstoday.com/articles/312045.php, MacGill 2018). Aggressive factors include *Helicobacter pylori*, HCl, pepsins, nonsteroidal anti-inflammatory drugs (NSAIDs), bile acids, ischemia, hypoxia, smoking and alcohol. While defensive factors include bicarbonate, mucus layer, mucosal blood flow, prostaglandins and growth factors (Harold *et al.,* 2007). Peptic ulcer disease (PUD) is an illness that affects a considerable number of people worldwide. The incidence of peptic ulcer has been shown to be common in Africa and South Asia (Wikipedia, 2011). When these ulcers occur in the stomach, they are called gastric ulcers but when they occur in the first portion of the intestine they are called duodenal ulcers. "Peptic Ulcer" is the term used to describe either or both of these two types of ulcers.

Peptic ulcer disease and its complications remain the cause of significant morbidity worldwide, representing a major burden for health care resources (Tanih *et al.,* 2010). Although potent anti-ulcer drugs are available, most of them produce several toxicities, thus emphasizing the need to search for new alternatives (Lavnya *et al*., 2012). As high as 80% of the world population depends on plant-derived medicines for the first line of

primary health care (Panda and Sonkamble 2012) reinforcing the theory that plant extracts can be good sources of new drugs.

Anatomy and Functions of the Stomach

The stomach produces hydrochloric acid which is a very strong acid. This acid helps to digest and break down food before it enters the small intestine (duodenum). The lining of the stomach is covered by a thick protective mucous layer, which prevents the acid from destroying the wall of the stomach.

Etiology of Peptic Ulcers

I *Helicobacter pylori infection*.

H. pylorus is a gram negative, microaerophilic bacterium found usually in the stomach. It was identified in 1984 by Australian scientists Barry Marshall and Robin Warren who found it present in person with chronic gastritis and gastric ulcers, conditions not previously believed to have a microbial cause (Marshall and Warren 1984). It is also linked to the development of duodenal ulcers and stomach cancer (Blaser, 2006). More than 50% of the world's population harbor *H. pylori* in their upper gastrointestinal tract and while the incidence is decreasing in developed countries, it is actually increasing in developing countries (Amieva, 2016). *H. pylori* are found in the mucus, on the inner surface of the epithelium and occasionally inside the epithelial cells themselves (Petersen and Krogfelt, 2003).

This bacterium which has a twisted spiral shape infects the mucous layer lining the stomach and produces an inflammation in the stomach wall called gastritis and it is believed to be probably acquired from contaminated food and drink (Wroblewski *et al.*, 2010). When H. *pylori* bacteria damage the protective mucous layer of the stomach, further damage by stomach acid gives rise to an ulcer. It takes advantage of the stomach's

own mucus for protection and any acid that does reach the bacteria is hydrolysed by *H. pylori's* urease enzyme in the following reaction:

Urea + stomach acid + water ⟶ bicarbonate + ammonia

$C{=}O{\cdot}2NH2 + H^{+} + 2H_20 \longrightarrow HCO_3 + 2NH_4^{+}$

The products of this reaction, bicarbonate and ammonia, are strong bases that further protect the bacteria because of their acid-neutralizing capability **(**Scott *et al.,* 2010). Furthermore, the biochemicals produced by *H. pylori* like proteases and the ammonia produced in the above reaction is used by *H. pylori* to regulate pH, they are toxic to epithelial cells, disrupts tight junctions and cause apoptosis **(**Scott *et al.,* 2010)

II ***Inflammation and gastritis***

The inflammatory response caused by the *H. pylori* bacteria colonizing near the pyloric antrum induces G cells to secrete the hormone gastrin (Blaser and Atherton, 2004). The gastrin so secreted stimulates the parietal cells to secrete more acid into the stomach lumen and over time increases the number of parietal cells as well **(**Schubert and Peura, 2008*).* The increased acid load damages the stomach, which may eventually result in ulcers.

III ***Non-steroidal anti-inflammatory drugs (NSAIDs)***

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin have the ability to cause gastroduodenal ulceration and this effect is related to the ability of these agents to suppress prostaglandin synthesis (Lichtenberger *et al.,* 2007). In the stomach, prostaglandins play a vital protective role, stimulating the secretion of bicarbonate and mucus, maintaining mucosal blood flow and regulating mucosal cell turnover and repair. Thus, the suppression of prostaglandin synthesis by NSAIDs results in increased susceptibility to mucosal injury and gastroduodenal ulceration (Deore *et al.,* 2011). Another mechanism by which aspirin damages the gastric mucosa is the increased production of Nitric oxide (NO) (Konturek *et al*., 2006). NO is a mediator not only of

gastrointestinal mucosal defense **(**Bjorne *et al.,* 2004) but also of its damage (Weitzberg and Lundberg 1998). It has been shown that different concentrations of NO have completely opposite effects on the same tissue (Wallace *et al.,* 2000). Furthermore, independent of effects on prostaglandin synthesis NSAIDs can disrupt the layer of surface-active phospholipids on the mucosal surface. When NSAIDs associate with these surface phospholipids the hydrophobic barrier becomes hydrophilic allowing acid to permeate the mucosal lining resulting in disruption of mucosal integrity (Lichtenberger *et al.*, 2006).

IV *Stress*

Stress-induced ulcer is believed to be mediated by the release of histamine which increases gastric acid secretion (Siddiqui *et al.,* 2019). Physical or psychological stress is one of the common causes of upper gastrointestinal ulceration (Hoogerwerf and Pasricha 2006). Although the pathogenesis of gastric lesions due to stress is not completely understood, the production of oxygen free radicals via the xanthine-xanthine oxidase system and neutrophils and lipid peroxidation initiated by the produced reactive oxygen species (ROS) have been used to explain the mechanisms of acute gastric lesion formation associated with stress (Kumar, 2011)

V *Alcohol*

Ethanol is considered a risk factor for developing gastric ulcers. It readily penetrates the gastric mucosa due to its ability to solubilize the protective mucous thereby exposing the mucosa to the proteolytic and hydrolytic actions of hydrochloric acid and pepsin (Oates and Hakkinen, 1988). It also leads to an increase in lipid peroxidation, oxidative stress, leukotriene production, and generation of free radical resulting in cell and membrane damage (Mohod and Bodhankar 2011). It has been reported that leukotriene antagonist

and 5-lipoxygenase inhibitors are capable of inhibiting alcohol and NSAID-induced gastric ulceration in rats (Roy *et al*., 2013).

Symptoms of peptic ulcer

Small ulcers may not cause any symptoms however big ulcers can cause serious bleeding. There are common shared symptoms like feeling of fullness, burning sensation at the epigastric region, bloody or dark stools, an empty feeling in the stomach often 1–3 h after a meal and mild nausea (Malagelada *et al.*, 2007).

Diagnosis

The following tests could be done to diagnose peptic ulcer

1. Endoscopy: This involves the insertion of a thin tube with a camera on the end through the mouth into the gastro intestinal tract to see the stomach and small intestine. During an endoscopy, a biopsy may be taken from the wall of the stomach to test for H. *pylori.*

2. X-ray of the upper GIT: This can be taken after drinking a thick substance called barium.

3. Hemoglobin blood test to check if there is anemia.

4. Stool analysis to test if there is blood in the stool.

1.2 Statement of Problems

Worldwide, peptic ulcer disease is responsible for substantial premature mortality, with much of the burden in low income and middle-income countries (Global burden of disease study, 2013). Treatments of PUD are often associated with high relapse rates and limiting side effects from the drugs (Benjamin *et al.,* 2018). Studies from Nigeria by Bashir and Ali in Kano reported an *H. pylori* prevalence of about 81% (Bashir and Ali 2009). Furthermore, probably due to involvement of multiple factors in the etiology of peptic ulcer disease and *H. pylori* resistance to most antibiotics, it has not been possible to produce a single drug that can completely cure peptic ulcer disease (Gao *et al.,* 2010 and

Thung *et al.,* 2016). Moreover, peptic ulcer-healing drugs combinations for the eradication of *H. pylori* are expensive. Therefore relapse may also occur due to unaffordability. There is therefore the need to continue to search for a better peptic ulcer healing agents especially from natural source like plants since extracts from plants are known to exert their pharmacological activity through multiple mechanisms with little or no side effects (Laure *et al.,* 2017)

1.3 Justification and Significance of the Study

Since ancient time, plants and plant derived-products have been used in folklores around the world for the treatment of several ailments and diseases (Alexandra *et al.,* 2018). Nowadays, herbal medicine is becoming a viable alternative treatment over the commercially available synthetic drugs for peptic ulcer (PU) management and treatment (Keshavarzi *et al.,* 2014, Hashemi *et al.,* 2015). This is premised on its lower cost, perceived effectiveness, availability as well as little or no adverse effects. A number of these herbal remedies have demonstrated gastroprotective properties and have been used in the treatment of PU, digestive disorders and other related ailments for several centuries (Areej *et al*., 2017). Many tropical herbs have been scientifically reported to possess potent antiulcer activity (Tables 1 and 2). For example, the gel from aloe vera was found to have ulcer-healing effect, attributed to its effects on fibroblast growth factor and collagen formation (Hashemi *et al.,* 2015). The ethanol root extract of *Zizyphus oenoplia* (Rhamnaceae) exhibited anti-ulcerogenic activity with possible mechanism including increase in prostaglandin synthesis (Jadhav and Prasanna, 2011). *Persea americana* aqueous seed extract is being used in folklore medicine for the treatment of stomach ache and other ailments (Pamplora and Roger, 1999**)** without scientic proof to back this up, the study was therefore embarked on in order to provide justification for this use

Poor out-come and treatment failures are commonly associated with gastric ulcer management mainly as a result of resistance by *H. pylori* bacteria. Therefore, this study seeks a novel naturally occurring therapeutic product which may be cost effective and accessible with no relapse and serious side effects. In addition it is hoped this study will turn *P. americana* seeds into useful product instead of waste.

1.4 Aim and Objectives.

The aim of this study is to evaluate the ulcer-healing properties of aqueous seed extract of *Persea americana* and its fractions.

Specific objectives

The specific objectives of this study are to

1. Determine protective and curative effects of the extract and fractions on peptic ulcer using different models.
2. Determine the anti-microbial effects of the extract and fractions.
3. Identify and isolate the major compounds in the extract and fractions responsible for the anti-ulcer effects.
4. Elucidate the structure of the isolated compound

CHAPTER TWO

LITERATURE REVIEW

2.1 Pathophysiology of Peptic Ulcer Disease

Under normal conditions, a physiologic balance exists between gastric acid secretion and gastroduodenal mucosal defense. Mucosal injury occurs resulting to peptic ulcer when the balance between the aggressive factors and the defensive mechanisms is disrupted (Rao, *et al*., 2000). Aggressive factors, such as nonsteroidal anti-inflammatory drugs (NSAIDs), *H. pylori* infection, alcohol, bile salts, acid and pepsin, can alter the mucosal defense and subsequent epithelial cell injury (Valle *et al*., 2005). The defensive mechanisms include tight intercellular junctions, mucus, mucosal blood flow, cellular restitution and epithelial renewal (Valle *et al*., 2005). Furthermore, most patients with duodenal ulcers have impaired duodenal bicarbonate secretion which has also proven to be caused by *H. pylori* because its eradication reverses the defect (Sung *et al*., 2010). Duodenal colonization by *H pylori* was found to be a highly significant predictor of subsequent development of duodenal ulcers in one study that followed 181 patients with endoscopy-negative, non-ulcer dyspepsia (Pietroiusti *et al*., 2005)

2.2 Management of Peptic Ulcer Disease

Non-pharmacological approach

Endoscopy

Endoscopy is a procedure in which an instrument is introduced into the body to give a view of its internal parts. Endoscopy is commonly used in clinical practice to diagnose peptic ulcers and to exclude malignant ulcers and other conditions that could be causing dyspepsia. Endoscopy also has a large role to play in the treatment of PUD complications such as bleeding ulcers and pyloric strictures as described below (Kurata and Hail, 1984).

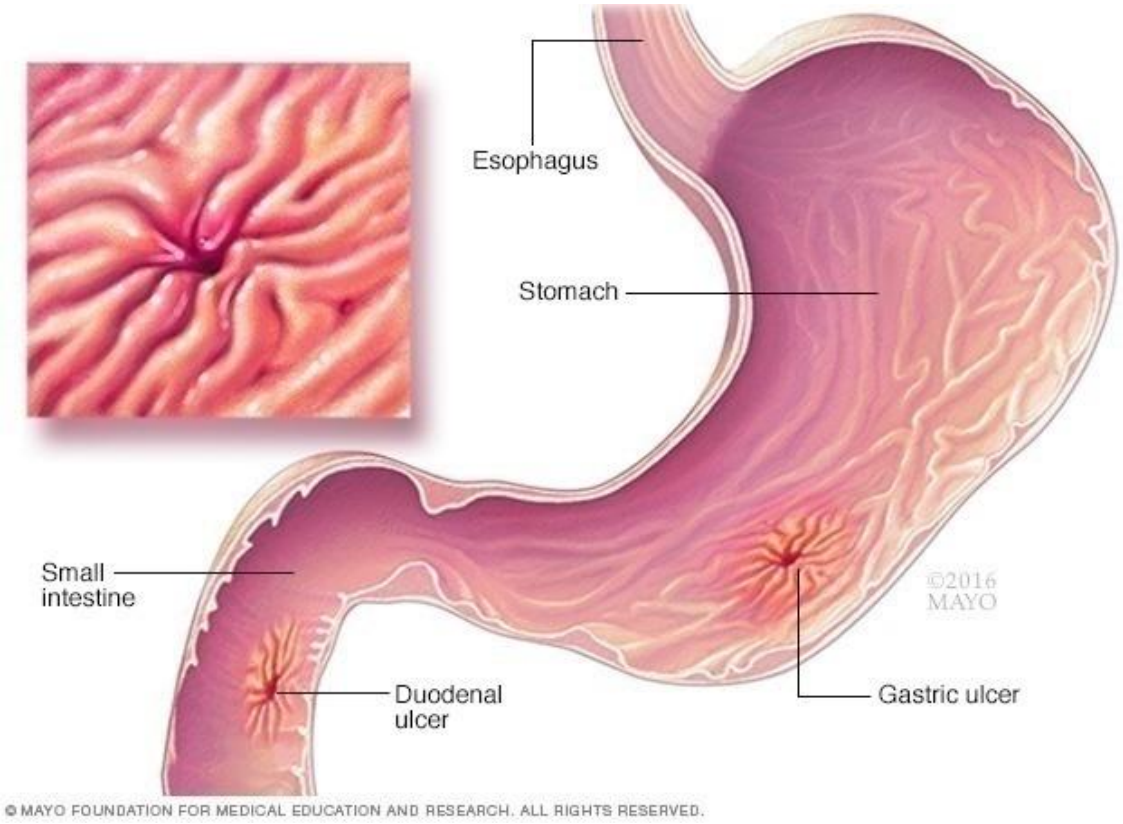

Figure1. Schematic diagram of Peptic Ulcer Disease Manifestations

Radiology

A double contrast barium study is a radiological technique which can easily detect ulcers both in the stomach and duodenum but it is only used where endoscopy is either technically difficult or where the patient prefers it to endoscopy. Endoscopy is preferred because it also allows biopsies and *H.pylori* testing.

Endoscopic therapy

Endoscopic therapy is superior to medical therapy in the treatment of bleeding ulcers, to achieve haemostasis and reduce rates of rebleeding. With the aid of endoscopy, adrenaline 1:10,000 mixed with saline is injected into the base of the ulcer where it causes vasoconstriction and mechanical plugging. Thermal coagulation of the bleeding vessel by using either a heater probe or argon plasma gas is another technique carried out using endoscopy and it is effective in high risk bleeders (that is those with a spurting vessel or visible vessel which indicates a recent bleeding). Ligation with small metal pins known as endoclips is also effective in high risk bleeders though this can be a technically difficult procedure. The combination of injection therapy with either endoclips or thermal coagulation achieves better results than one technique. Following endoscopic therapy, the rebleeding in high risk bleeders is around 20% in the first 48 to 72 hours. Effective endoscopic therapy reduces the need for surgery in peptic ulcer bleeding

Surgery in peptic ulcer treatment

Surgical treatment of peptic ulcer is reserved for ulcer disease unresponsive to medical management or emergency treatment of complications, such as bleeding. In some cases an elective surgery to decrease stomach acid secretion may be necessary.

Adopting changes in life styles

Avoidance of spicy food and fatty food is usually recommended as those generally irritate stomach lining. Patients are usually advised to moderate or avoid smoking, alcohol

beverages and pain relievers such as NSAIDS. The relief of anxiety and stress are also recommended since stress triggers the release of histamine which increases the rate of gastric acid secretion (Kitagawa *et al*., 1979).

Pharmacological Treatment

Classes of drugs for peptic ulcer treatment, mode of action and adverse effects

Drugs Reducing Gastric Parietal cell Stimulation in the Primary Level of Acid Secretion Process

Therapeutic strategy for treating ulcer at the molecular level generally involves reducing acid secretion by inhibiting receptors/mediators at the initial level, intermediate level and final level of acid secretion. In the initial level, the strategy aims to reduce secretion by preventing stimulation to transmitters including histamine, acetylcholine and gastrin. The intermediate level mainly involves interference on the role of carbonic anhydrase in promoting acid secretion. In the final stage is the proton pump (H+ K+ ATPase) which has been the target for inhibition to reduce acid secretion.

Acetylcholine inhibitors

Pfeiffer *et al*. (1995) suggested that secretion of acid, mucus and pepsinogen in the gastric mucosal is stimulated via muscarinic receptors. Over expression of M_3 receptors in duodenal ulcer patients is proved by auto radiographic techniques; thus blockade of this receptor subtype will reduce the pain by decreasing the duodenal motility and provide an effective anti-secretory therapy. Based on the high affinity to block the muscarinic receptors on the intramural ganglia of stomach wall, pirenzepine was developed as an anti-secretory drug, which was followed by telenzepine, a more potent derivative with improved healing rates (Dammann *et al*., 1989). Parasympathetic side effects (Lazzaroni *et al*., 1986) of these agents include dry mouth, blurred vision and constipation. These side effects along with their incomplete inhibition of gastric acid secretion limit their clinical use.

Histamine inhibitors

H_2 receptor antagonists competitively inhibit the interaction of histamine with H_2 receptors thereby reducing both volume and H+ ion concentration of the gastric juice. They are selective and have little or no effect on H_1 receptors. They also inhibit acid secretion elicited by gastrin, muscarinic agonists, food, sham feeding, fundic distension, as well as other pharmacological agents. They also inhibit basal and nocturnal acid secretion. These effects contribute in a major way to their clinical efficacy. Black *et al.,* (1972) identified H_2-receptor and prototype H_2-receptor antagonist, burimamide. The potency of burimamide at inhibiting gastric acids secretion far exceeded that produced by anti-cholinergic drugs and was devoid of side effects. However, it had poor bioavailability hence it was subsequently replaced by metiamide (Wyllie *et al.,* 1972) which also because of its side effects like agranulocytosis, was withdrawn from the clinical trials.

Price *et al*., (1978) reported cimetidine as the third H_2 receptor antagonist to be tested in humans which was similar to metiamide in its pharmacological profile, but did not cause agranulocytosis. Discovery of this molecule reduced the necessity of surgical procedures for peptic ulcer diseases. Further, ranitidine (Roberts and McDonald, 2003) was introduced as more potent drug with a much superior safety profile. Third and most potent antagonist was famotidine, being 20-50 times more potent than cimetidine and 6-10 times more potent than ranitidine (Najm 2011). It was also used once daily. Nizatidine reported by Berardi *et al.,* (1988) followed famotidine. Each of these drugs are rapidly absorbed and eliminated after oral administration. H_2 receptor antagonists are generally extremely safe drugs with incidence of adverse effect of cimetidine less than 3%. Adverse effects include dizziness, nausea, skin-rashes, somnolence, confusion, impotence, gynecomastia, hematological effects and altered function of immune system. Rarely do

they cause bone marrow depression, hepatitis, and anaphylaxis. However in chronic as well as acute cases of ulceration, their effectiveness is far from satisfactory. Because of this, they are increasingly replaced by proton pump inhibitors.

Drugs Reducing Carbonic Anhydrase (CA) Activity in the Intermediate Level of Acid Secretion

The involvement of CA I and CA IV in gastric acid secretion, effect of CA inhibitors in reducing HCl secretion and their healing effect on gastric and duodenal ulcers are well documented (Puscas *et al*., 1999). In vivo results, performed in humans, show that omeprazole inhibits not only H+/K+ ATPase, but also CA II and CA IV isozymes present in large quantities in the cytosol, in the walls of the secretory canaliculi, and in the parietal cell membrane. Further, gastric acid secretion is inhibited in humans after oral administration of acetazolamide in therapeutic doses of 25 mg/kg of body weight (Puscas *et al*., 1999). Acetazolamide exhibit antiulcer action in acute experiments because of inhibition of CA-II, but its effect on Gastric ATPase is not clear (Puscas *et al*., 1999).

Drugs Inhibiting Gastric ATPase in the Final Level of Acid Secretion

Proton pump is the ultimate mediator of gastric acid secretion by parietal cells (Okamoto and Forte 2001).With the identification of H+/K+-ATPase as the primary gastric proton pump, it was proposed that activation of H+ secretion occurred by incorporation of H+/K+-ATPase rich tubulovesicles into the apical plasma membrane and the pumps were re-sequestered back into the cytoplasmic compartment on return to the resting state. Currently this is achieved by blocking the irreversible H+/K+-ATPase. They are popularly referred as Proton Pump Inhibitors (PPIs). They bind to the gastric proton pump on the parietal cell membrane, inhibiting the release of hydrogen ions from the parietal cells into the lumen of the gastric glands and hence stomach (Ife *et al*., 1989).

Inhibition of H+/K+-ATPase as a means of controlling gastric pH has attracted considerable attention in recent years with the discovery of benzimidazole sulfoxide class of antisecretory agents. Timoprazole, as one of the first well-defined inhibitor of gastric proton pump (Ruwarte *et al*., 1984), was followed by more potent picoprazole and omeprazole (Lindberg *et al*., 1990). Clinically used PPIs include Omeprazole, Lansoprazole, Rabeprazole, Pantoprazole and Esomeprazole. The draw backs relating to use of irreversible proton pump inhibitors includes extreme acid suppression which sometimes leads to achlorohydria at recommended doses and that may produce enteric infections like typhoid, cholera, and dysentery (Jain *et al.,* 2007). Other side effect includes abdominal pain, diarrhea, nausea and headache.

Drugs with other Mode of Action

Antacids

Naturally occurring carbonates, potash, bismuth are currently widely used. Antacids neutralize HCl to form water and carbon dioxide. Hydroxides of aluminum and magnesium are the most common constituents of antacid preparations. Sodium bicarbonate, calcium carbonate are also used with silicates and phosphates. Simethicone, a surfactant that may decrease foaming and thus, esophageal reflux, is part of many antacid preparations. However, use of antacid is limited to acid neutralization only and does not affect hyper acid secretion. Besides they suffer from other limitations. Sodium bicarbonate, which is used as a systemic antacid causes alkalosis. Magnesium, aluminum and calcium salts act locally and are less likely to develop alkalosis, but magnesium salts cause diarrhea, whereas aluminum and calcium salts cause constipation

Anti H. pylori drugs

Double or triple antimicrobial therapies, in combination with antisecretory drugs, are useful against *H. pylori* infection and associated peptic ulcers. Bismuth compounds are

also being included in regimen probably due to their cytoprotective action. Triple therapy with metronidazole, a bismuth compound and tetracycline, amoxicillin or clarithromycin for two weeks is recommended for treatment of *H. pylori* infections. However, the therapeutic limitations of this triple therapy include complex regimen, high cost for treatment and related nausea, diarrhea and dizziness (Labenz, 2000).

Prostaglandin analogues

There are a number of prostaglandin analogues like arbaprostil, enprostil, misoprostol, rioprostil and trimoprostil. They are inferior to H_2-receptor antagonists as regards their effects on ulcer healing, pain relief, and relapse prevention and less effective than expected in acid inhibitory action. They also possess cytoprotective property. They may prove useful as replacement therapy in patients requiring nonsteroidal anti-inflammatory drugs (Laursen *et al*., 1989)

Mucoprotectives

Sucralfate, a basic aluminum salt of sucrose, was the first successful drug with a major cytoprotective mechanism of action. It binds bile acids and pepsin and adheres to both ulcerated and nonulcerated mucosa. Sucralfate stimulates the synthesis and release of gastric mucosal prostaglandins as well as bicarbonate and the epidermal growth factor which stimulates healing (Jensen and Funch 1992). Bismuth is another compound that has shown efficacy against two major gastrointestinal disorders: peptic ulcer disease and diarrhea. In peptic ulcer disease it is as effective as the H_2-receptor antagonists, costs considerably less, and offers a lower rate of relapse. When *Helicobacter pylori* are implicated, bismuth acts as an antimicrobial agent, suppressing the organism but not eliminating it. In some studies, bismuth compounds have been used with conventional antibiotics, producing elimination of the organism, (Gorbach, 1990 and Najm, 2011).

Table 1 Some Plants with Anti-Ulcer Activity

S/N	Plant	Part of plant	Solvent	Ulcer model	Refrence
1	*Calotropis procera*	Leaf and fruit	Hydro ethanol	Ethanol	Areej *et al*., 2017
2	*struthanthus marginatus*	Leaf	Ethanol	Ethanol	Danilo da Cruz *et al.*, 2018
3	*Jasmine grandiflorum*.	Root and leaf	ethanol	Pyloric	Hunasagi *et al.*, 2018
4	*Aframomum pruinosum*	Seeds	Water	Indomethacin	Laure *et al.*, 2017.
5	*Cydonia oblonga* (Quince)	Fruit	Hydroethanol	Indomethacin induced	Morteza *et al.*, 2017
6	*Cibotium barometz*	Leaf	Methanol	Stress	Nahla *et al.*, 2017
7	*carica papaya*	Unripe fruit	Hydroalcoholic extract	Pyloric ligation	Ramandeep and Kalyan 2017
8	*Beta vulgaris*	Root	Ethanol	Pyloric ligation	Samyuktha *et al.*, 2017
9	*Acacia Arabica*	Gum	Methanol	Stress	Omayma *et al.*, 2011.
10	*Aloe vera*	Leaf	Water	Ethanol	Borra *et al*., 2011)
11	*Psidium guyava*	Leaf	Methanol	Ethanol	Uduak *et al.*, 2012
12	*Mangifera indica*	Flower	Water	Pyloric ligature	Neelima *et al.*, 2012
13	*Annona squamosa*	Leaf	Water	Pyloric-ligation	Mohamed Saleem, 2012.

Table 2 Some Plants with Anti-H.pylori Activity

S/n	Plant	Part of plant	Refrence
1	The aqueous extract of *Enantia chlorantha* (*Annonaceae*)	stem bark	Tan *et al.,* 2010
2	*Nigella sativa* L. (*Ranunculaceae*)	Seeds	Salem *et al.,* 2010
3	*Impatiens balsamina* L	Root, stem, leaf, seeds and pod	Wang *et al.,* 2009

2.3 Literature Review on *P. americana* Mill

Persea americana Mill is from the plants kingdom, (angiosperms), subkingdom of tracheobionta (vascular plants) and superdivision of spermatophyta (seed plants). It is from the division of magnoliophyta (flowering plants) and of the order Laurales. The family is *Lauraceae* and of the genus, *Persea* Mill. It belongs to the class magnoliopsida (dicotyledons) while the bionomial name is *Persea americana* Mill. There are eight species of *Persea americana* namely, *Persea americana* Mill, *Persea borbonia, Persea humilis, Persea krugii, Persea nubigena, Persea palustris, Persea schiedeana and Persea urbaniana.*

The fruit is commonly referred to as avocado pear, alligator pear and butter fruit. It is known as Ebenmbakara in Ibibio, Ube bekee in Igbo and Ado in Yoruba. It is a widely distributed plant in the lowlands and rain forest areas of Nigeria. The leaves are thick, glossy, dark green above and paler below and are briefly shed around the time of flowering while the trees are partially self-pollinating. The fruit is a large berry containing a single large seed known as “pit" or “stone". *P. americana* trees are cultivated in tropical and Mediterranean climates *(*Morton, 1987*).*

Traditional uses

This plant has been used traditionally by man for a long time for the treatment of various ailments. The root, bark, fruit, seed and leaf are used extensively in traditional medicine in many tropical and subtropical countries for the treatment of various ailments (Owolabi, *et al*., 2005). Its seed is used in the treatment of diarrhea, dysentery, toothache, stomach ache and intestinal parasites (Pamplora and Roger 1999). The aqueous seed extract are used externally for treatment of dandruff on the hair and for skin beautification (Pamplora and Roger 1999). The leaves have been popularly used in the treatment of diabetes in countries of Latin America and Africa (Lima *et al*., 2012). The fruit is employed as a

vermifuge and remedy for dysentery. The leaves decoction is also taken as a remedy for diarrhea, sore throat, hemorrhage and to stimulate and regulate menstrual period (Morton 1987).

Phytochemical study

Omodamiro *et al* (2016) reported the presence of alkaloids, flavonoids, saponins, steroids, tanins, and phenol as phytoconstituents in *Persea americana* fruit. In another study Idris and his colleagues carried out preliminary phytochemical analysis of the aqueous seed extract. Preliminary phytochemical screening revealed the presence of flavonoids, saponins, tannins, steroids alkaloids and terpenoids (Idris *et al.,* 2009). The presence of monounsaturated fatty acids were reported by Akpabio *et al* (2011). Del Refugio *et al* (2004) reported the elucidation of two glucosylated abscisic acid derivates from avocado seeds. The two glucosylated abscisic acid derivates were isolated and identified as (1′*S*,6′*R*)-8′-hydroxyabscisic acid β-d-glucoside and (1′*R*,3′*R*,5′*R*,8′*S*)-*epi*-dihydrophaseic acid β-d-glucoside (Del Refugio *et al.*, 2004).

2.3.1 Pharmacological activities of *P.americana*

The anti-oxidant property

Using the radical scavenging assay methods the anti-oxidant property of avocado seed oil and the power of the avocado seed extract (ASE) to delay oil oxidation by oxidation induction time were measured with differential scanning calorimetry (DSC). The individual contribution of each of the compounds present in the extract was analyzed. The sum of all of them contributed up to 84 % of the total radical scavenging activity. The concentration of 0.75 % avocado seed extract caused a delay in the oxidation that is close to 80%, as measured by oxidation induction time (Francisco *et al.,* 2018). Measurement of antioxidant activity using DPPH (1,1-Diphenyl-2-picrylhydrazyl) method on avocado leaf extract and vitamin C (control group) revealed the antioxidant activity of avocado

starch extract (absorbance DPPH = 0.797) to be IC50 of 72.61 mg L^{-1} while vitamin C the positive control was IC_{50} of 23.03 mg L^{-1} (Nurdin *et al.,* 2018). In another study, the antioxidant activity of avocado seed was investigated using DPPH, superoxide, and hydrogen peroxide scavenging assays. Antioxidant activity assays showed seeds from ripe avocado pears exhibited higher free radical scavenging ability with lower IC 50 values relative to the unripe seeds (Alagbaoso *et al*., 2015). The antioxidant properties of *Persea americana* Mill seed was also demonstrated using four different extraction solvents at different dilution ratios. The results showed the seed contains considerable amounts of phenols, flavonoids and pro-anthocyanidin which contributed to the antioxidant capacity of seeds (Aderibigbe *et al.,* 2016). The Bioactive characterization of the hydro ethanolic extracts of peels and kernels of *Persea americana* fruit were carried out with regard to their individual phenolic profile by HPLC-DAD/ESI –MS. This was correlated with their antioxidant, antimicrobial and cytotoxic activities. Avocado byproducts presented a very distinct phenolic profile, with higher concentration in peels (227.9 mg/g of extract for total phenolic content). Although the kernels showed a great antioxidant potential (EC50 values ranging from 18.1 to 276 μg/mL), peels presented the highest potential (EC50 ranging from 11.7 to 152 μg/mL) with an overall better performance in the antibacterial assays (Bruno *et al*., 2018).

The anti-motility effects

In another investigation Odo *et al.* reported, at 200 mg/kg body weight, the chloroform and methanol fractions produced significant ($p<0.05$) and dose-related decreases on gastro-intestinal motility and decrease in concentration of the intestinal fluid potassium ions. The results of the fractions were comparable with those of the standard anti-diarrhoeal drug, hyoscine butylbromide (3 mg/kg body weight) (Odo *et al.,* 2013).

The toxicity effects

Experiments were conducted by Ozolua *et al* (2009) to determine the oral median lethal dose (LD_{50}) and other gross toxicological manifestations on acute basis**.** In the sub-acute experiments, the animals were administered 2.5 g/kg (p.o) per day of the seed extract for 28 consecutive days. Animal weight and fluid intake were recorded during the 28 days period. Terminally, kidneys, hearts, blood/sera were obtained for weight, haematological and biochemical markers of toxicity. Results show that the LD_{50} could not be determined after a maximum dose of 10 g/kg. Sub-acute treatment with the extract neither affected whole body weight nor organ-to-body weight ratios but significantly increased the fluid intake ($P < 0.0001$). Haematological parameters and the levels of ALT, AST, albumin and creatinine were not significantly altered. However, the concentration of total proteins was significantly increased in the treated group. Odoh *et al,* (2013) and Eduardo *et al* (2013) reported the LD_{50} value of the seed extract to be lower than 5000 and 1200.75 mg/kg/bw respectively. On the other hand, Eduardo *et al* (2013) documented the genotoxicity of the seed extract at 250 mg/kg; the genotoxicological study of the effect on peripheral blood cells revealed no *In vivo* mutagenicity (Eduard *et al.*, 2013).

The anti- microbial effects

Antimicrobial activity of the seed extracts against *Escherichia coli, Staphylococcus aureus* and *Salmonella typhi* was carried out using the disc diffusion techniques. The petroleum ether extract exhibited activity on all the test organisms, producing zones of inhibition ranging from 5-14 mm while the ethylacetate extract produced zones of inhibition ranging from 14-30 mm. The activities of methanol and chloroform extracts were potent only against *S.aureus* (31 mm and 27 mm) while that of ethyl-acetate was least

Figure 2.The leaves, Fruits and Seed of *Persea americana* Mill (Laureceae)

against *E.coli* (5 mm). However *S.typhi* and *E.coli* were resistant to chloroform and methanol extracts (Ilozue *et al*., 2014). In another study the evaluation of the antibacterial efficacy of the crude ethanolic and aqueous extract of the fruit peel of *Persea Americana* against selected clinical isolates using agar well diffusion method was carried out. The result of the antibacterial screening revealed the sensitivity of the bacteria to ethanol extract in this order *E. coli* > *S. typhi* > *P. vulgaris* with mean zones of inhibition ranging from 6 to 14, 3 to 15 and 3 to 8 mm respectively. For the aqueous extract; *S. typhi* > *P. vulgaris* > *E. coli* with mean zone of inhibition ranging from 0 to 15, 0 to 4 and 0 mm respectively. The general results of the evaluation considered the plant as a potential source of novel antibacterial agents against *Enterobacteriace*a and ethanol, a better extracting solvent (Michael and Charles, 2016).

Hepato-protective effects

The evaluation of the hepatoprotective and haemopoietic activity of *Persea americana* seed was carried out using seven groups of wistar albino rats. Group A served as normal control, group B served as experimental control while group C-G served as test group administered different doses (500, 250, 125, 62 and 31 mg/kg) of *P. americana* seed extract for 14 days. The serum concentration of alkaline phosphatase, asparate amino transferase, alanine aminotransferase, direct and total bilirubin were determined spectrophotometrically while the haemopoietic effect was evaluated by carrying an assay on the haemoglobin, packed cell volume and total red blood cells. Reduction in liver enzymes and increase in haematological parameters in the test group indicated *P. americana* seeds have hepatoprotective and haematological properties (Omodamiro *et al.*, 2016).

The anti-inflammetry effects

Adeyemi *et al* reported the effect of 1600 mg/kg (p.o) of aqueous leaf extract of avocado on acetic acid induced writhing in mice. There were 57 % reduction in writhing and 87.2 % increase in the threshold of pain upon administration of 800 mg/kg as noted by the hot plate test and the inhibition of both phases of formalin induced pain in a dose-dependent manner (Adeyemi *et al.,* 2002). The anti-inflammatory activity and antigenotoxic effect of hydroalcoholic leaf extract of P. *americana* in albino Wistar rats against whole body X-ray irradiation was carried out by Amith *et al.,* (2017) at oral doses of 25, 50, 100, 200, and 400 mg/kg body weight of leaf extract for five days. This was followed by whole body exposure to X-rays of 8 Gy. The leaf extract offered significant ($P<0.05$) protection to rats from whole body exposure to X-rays and helped in antagonising the radiation effects, thereby combating acute-radiation induced damage in living systems (Amith *et al.,* 2017).

The anti-diabetic effects

The anti-diabetic and protective effect of different concentrations (20, 30, 40 g/L) of the hot aqueous seed extract on alloxan-induced diabetes has been reported (Anthonet *et al.,* 2013). In another study Alhassan *et al* (2012) reported the effect of the aquous seed extract at 400 mg, 800 mg and 1200 mg/kg on alloxan induced diabetes. A significant decrease ($P<0.001$) in blood glucose were observed in all groups compared to control and a significant increase in blood glucose ($p<0.05$) was observed one week after withdrawal of the extract.

The anti-hypertensive effects

The investigation of the effects of the aqueous seed extract (AE) on the mean arterial pressure (MAP) and Heart Rate (HR) of naïve and 260 mg/kg/day x 1 0 days pretreated rats has been carried out. Naïve rats were given bolus injections of (a) - AE (240, 260,

280 mg/kg); (b) 2 µg/kg of acetylcholine (ACh) + 240, 260, 280 mg/kg of AE; or bolus doses of ACh (1, 2, 4 µg/kg). The 10-day pretreatment significantly reduced MAP (1 25.7 ± 1 1 .2 vs 92.1 ± 8.5 mm Hg) and HR (274.6 ± 39.3 vs 1 61 .6 ± 1 1 .6 beats/min). The effects of AE on MAP were comparable with those of Ach (Ozolua *et al*., 2009). In another study Imafidon and Amaechina (2010) documented the effect of the aqueous seed extract on sodium chloride (NaCl) induced hypertension. After the administration of the seed extract orally at doses 200 mg to 700 mg/kgbw for 4 weeks, blood pressure was reduced at all doses as well as reduction in plasma levels of triglycerides(TG), plasma total cholesterol (TC) and low density lipoprotein (LDL) at doses of 500 mg/kg and above. The effect of ethanolic extract of avocado pear seeds (ASE) on normal and monosodium glutamate (MSG)-compromised rats' kidney histology and some serum bio-functional parameters has been reported. The study demonstrated MSG-induced adverse effect on the rats' kidney histology and some serum bio-functional indicators (Anthony *et al.,* 2017).

Solid formulation of the seed extract

Formulation of the ethanol seed extract of *Persea americana* into tablet dosage form by direct compression has been carried (Majekodunmi and Ekpeyong, 2012). In another study a compound known as persin, isolated from the leaves was used to carry out the induction of apoptosis in human breast cancer cells (Butt *et al*., 2006).

Though a lot of research work has been done on the seed extract of P. *americana* mill, the evaluation of the ulcer-healing property is yet to be carried out. The need to fill this gap neccessited this study

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant materials

Sample collection and identification

The fruits of *P. americana* were obtained from Oye Nimo, Nimo, Njikoka local government area Anambra State, Nigeria in the fruiting season of April 2016. It was identified and authenticated by Dr J. E. Amadi, a Taxonomist at the Department of Botany, Nnamdi Azikiwe University Awka, Nigeria and a herbarium specimen, NAUH.13 was kept in the hebarium.

3.1.2 Drugs

Aspirin (Bayer Pharmaceutical Limited, UK), injection Atropine sulphate USP (Liveath Biopharma India), Cimetidine (Arochem Enterprises, New Delhi India), Histamine powder (≥ 97.0 %) (Sigma-Aldrich), Acetylcholine (Sigma-Aldrich**),** Adrenaline acid Tartrate (Transo-Pharm Handels GmbH DE Germany) were used.

3.1.3. Instruments

Water-bath, animal cages (3ft by 2ft by 2ft)**,** rotary evaporator, Mesh 60-200, UV Spectrophotometer (DU^R 6501 Beckman, U.S.A.), Binatone blender (Model BLG-401, China), microhaematocrit centrifuge (Beckman, U.S.A), Data capsule Evo 17304 (Ugobasile, Italy), isolated organ bath 4400 (Ugobasile, Italy), computer with software labscribe 2 (Ugobasile, Italy), Aerator pump SB-108 (Ugobasile Italy), Isometric transducer Evo 13062 (Ugobasile, Italy). Analytical HPLC (P580A LPG Dionex, Italy) comprising pump UVD 340S detector (photodiode array detector, Italy), ASI-100 auto sampler, column oven STH 585 and Dionex column (Eurosphere 100-C18, Italy) with integrated pre-column and chromelon 6.30 software (Italy), Vacuum liquid chromatography (VLC) chamber and thin layer chromatography (TLC) plate.

3.1.4 Chemicals and reagents

10 % Formaldehyde (Arihant chemical, India)**,** 20 % sodium hydroxide 0.4N (Green Chem Industries, USA)**,** Meryer's reagent (ACS Chemicals, India)**,** Wagner's reagent (ACS Chemicals, India)**,** N-hexane analytical grade (Zigma, India), 96 % v/v ethanol analytical grade (Zigma, India), Aluminium chloride solution (JHD, China), Fehling's solution (JHD, China), 20 % dilute sulphuric acid (Zigma, India), Sodium carboxyl methyl cellulose (Zigma, India) and tragacant powder (ACS Chemicals, India).

3.1.5 The Animals

The study was carried out using adult Albino rats (200 to 220 g) of both sexes bred locally in the Animal House of the Department of Pharmacology & Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria Nsukka, Nigeria. The Rabbits (1 to 2.5 kg), Guinea pigs (300 to 450 g) and Mice (25 to 35 g) were also purchased from Animal House of the Department of Pharmacology & Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria Nsukka. A total number of 360 Albino rats, 2 Rabbits, 8 Guinea pigs and 40 Mice were used. The Rats and Mice were fed with feed pellets, Top Feed (by Premier Feed Mills Sapele, Delta state, Nigeria) while the Rabbits and Guinea pigs were fed on green grasses predominantly *Panicum maxima* Jacq (Poaceae). The animals were given food and water *ad libitum* throughout the experiments. They were kept in specially constructed cages to prevent coprophagia during and after the experiment. The Rabbits, Guinea pigs and Rats of either sex were used for the in vitro study. All animal experiments were conducted in compliance with NIH Guide for Care and use of Laboratory Animals (Pub. No. 85-23 Revised 1985) as approved by the Nnamdi Azikiwe University, Awka Ethical Committee for the use of Laboratory Animals.

3.2. Methods

3.2.1. Extraction

The seeds of *P. americana* were removed from the fruits and chopped into small pieces, shade-dried for 5 days and grounded into fine powder with Binatone blender (Model BLG-401). The powder (I kg) was soaked in 2 litres of distilled water for 24 h. This was first filtered by passing it through a cotton plug and further filtered with filter paper (Whatman filter paper, No 1). The aqueous seed extract of *Persea americana* (ASEPA) was freeze-dried to a constant weight and stored at 4 °C in a amber-colored bottle until required for experiments.

3.2.2 Fractionation

To 100 g of freeze-dried ASEPA in a mortar, 100 ml of distilled water was added and mixed thoroughly. This was then poured into a separating funnel and another 100 ml of distilled water was added and mixed by shaking vigorously. Then, 250 ml of n-hexane was added and used to wash the extract by shaking vigorously. After 30 minutes the n-hexane layer was collected into a beaker. Subsequently, 250 ml volumes of n-hexane were added until the n-hexane layer became clear. The residue which was the water fraction was then removed from the separating funnel and the two fractions dried to a constant weight using rotary evaporator at 40^{o}C.

3.2.3 Acute toxicity study (LD_{50})

Acute toxicological study of the extract was carried out using the method described by Miller and Tainter (1944). Six groups (n=5) of male and female rats, starved for 24 h were administered orally with either distilled water (10 mg/kg) or ASEPA at the doses 250, 500, 1000, 2000 and 5000 mg/kg using an orogastric tube. After treatment the rats were fed with Top feed and water and observed for obvious signs of toxicity and mortality at hourly intervals for 24 h. Thereafter, for a total of 14 days (Bruce, 1985).

3. 2. 4 Phytochemical screening of the extract and fractions

Qualitative and quantitative phytochemical screening of ASEPA and fractions were carried out according to the procedures outlined by Harborne (1998) to determine the presence and concentration of the secondary metabolites.

Determination of median effective dose (ED_{50})

Five groups (n=5) of rats were used. The groups were given orally 200, 400, 600 and 800 mg/kg of ASEPA, while the negative control group received distilled water (10 ml/kg). The rats were treated for 14 days and starved with only access to water for 24 h before the induction of ulcer with a single-dose oral administration of absolute ethanol (96 %v/v, 1 ml/200 g). The ulcer indexes for all the five groups were determined using method described by Nyarko *et al.*, (2005). The reponse (% protection) was plotted against dose and by extrapolation the dose that produced 50 % ulcer protection (ED_{50}) was determined and the approximate value used to carry out this study. Applying the same method the ED_{50} of n-hexane fraction (NF) and water fraction (WF) were also determined using 200, 400 and 600 mg/kg doses of each fraction.

3.2.5 Anti-ulcer Activity

The ulcer-protective activity of the extract and fractions were evaluated using ethanol, aspirin, pyloric ligation and stress-induced ulcer models while the ulcer-healing effect was studied on ethanol, aspirin and stress-induced ulcer models. The animals were grouped into 8 (n=5) and treated orally as follows:

Group 1: 10 ml/kg distilled water (nagative control)
Group 2: Cimetidine 150 mg /kg (positive control)
Group 3: ASEPA 250 mg/kg
Group 4: ASEPA 500 mg/kg
Group 5: NF 250 mg/kg
Group 6: NF 500 mg/kg
Group 7: WF 250 mg/kg

Group 8: WF 500 mg/kg

3.2.5.1 Effect of ASEPA and fractions against ethanol-induced ulcer.

The method described by Nyarko *et al.*, (2005) was adopted. The rats were grouped as stated in section 3.2.5 and given daily, a single oral treatment of the doses as stated above in section 3.2.5 for 14 days, and then starved for 24 h with access to water. At the end of 24 hours absolute ethanol (96 % v/v) was administered (1 ml/200 g p.o) as a single dose to each animal and the animal sacrificed 1hr later. Macroscopic evaluation of the glandular portions of stomach was made by opening the stomach along the greater curvature, rinsed under a stream of water, pinned flat on a corkboard and viewed macroscopically with a hand lens (magnification x10). Each stomach was given a severity rating as stated by Ganguly and Bhatnagar (1973). The degree of ulcer protection for each treatment group was calculated as a percentage with respect to the mean ulcer index of the negative control group.

Normal stomach..... (**0**)
Red coloration..... (**0.5**)
Spot ulcer.....……. **(1)**
Hemorrhagic streak... .(**1.5**)
Ulcers.....….. **(2)**
Perforation.....…. **(3)**
Ulcer index (UI) and percentage protection were calculated as shown below;

$$UI = \frac{US}{UN} \times 10^{-1}$$

Where,

UI= Ulcer Index; UN = total number of ulcers per animal;

US = total number of severity score for each animal.

% Protection = Control mean ulcer index - Test mean ulcer index x100

Control mean ulcer index

3.2.5.2 Effect of ASEPA and fractions against aspirin-induced ulcer.

The method described by Nyarko *et al.*, (2005) was adopted. The rats were grouped and treated as previously described in section 3.2.5. The ulcers were induced with Aspirin (150 mg/kg p.o) and the animals sacrificed 4 hrs later. The degree of ulcer was recorded using method described in section 3.2.5.1.

3.2.5.3 Effect of ASEPA and fractions against pyloric ligature-induced ulcer.

The method used was as described by Shay *et al* (1945). The rats were grouped and treated as stated in section 3.2.5. Under light ether anesthesia, the abdomen was opened by a small midline incision below the xiphoid process; the pyloric portion of the stomach was slightly lifted and ligated, avoiding traction to the pylorus or damage to its blood supply. The stomach was then replaced carefully and the abdominal wall closed by interrupted sutures. After 4 h of pyloric ligation, the animals were humanely sacrificed with excess of anesthetic ether and the stomach dissected out. The gastric content was drained into a graduated tube to determine the volume, while the pH of the gastric juice was recorded using pH meter. The free acidity and total acidity were determined as stated below. The ulcer index and percentage protection were determined using the method previously stated in section 3.2.5.1.

Measurement of gastric acidity.

The gastric juice was centrifuged at 1000 rpm for 10 minutes. Then, 1 ml of the gastric juice was transferred into a porcelain dish, and two drops of Topfer's reagent was added to this and colour change appeared indicating the presence of free acid. Into this was added 2 drops of phenolphthalein indicator. This was titrated against 0.01N NaOH in a burette. When the trace of the red colour disappeared and is replaced by a canary yellow colour, the reading was taken from the burette and used for the calculation of the free acidity (the"active" acid). The titration continued until the color of phenolphthalein

appeared. The titration continued up to the point that further addition of the alkali does not deepen the pink color. The quantity of 0.01N NaOH from the beginning was noted and used for the calculation of the total acidity. This experiment was done in triplicate and the average taken (Ganguly *et al.*, 1973).

Calculation

$$\text{Acidity(x)} = \frac{\text{Volume of NaOH x Normality of NaOH} \quad 100}{0.1} \quad mEq/L$$

X= the acidity (free or total)

3.2.5.4 Effect of ASEPA and fractions against stress -induced ulcer

The method described by Alpine and Ward (1999) was adopted and the animals were grouped and treated as presented in section 3.2.5. After 24 hours fasting, stress ulcers were induced by forced-swimming the rats in the glass cylinder (height 45 cm, diameter 25 cm) containing water to the height of 35 cm maintained at 25 °C for 3 hrs. The animals were humanely sacrificed after 3 hrs period using excess anaesthetic ether and the stomachs excised and opened along the greater curvature. The degree of ulcer was recorded with the aid of hand lens (x10) and the ulcer grading was carried out as described in section 3.2.5.1

3.2.5.5 Healing effect of ASEPA and fractions.

The healing effect of ASEPA and the fractions were tested on ethanol, aspirin and stress-induced gastric ulcer as described by Hora and Okobe (1985). In each model animals were grouped as stated in section 3.2.5 and fasted for 24 h with access to water. The animals were then induced with ulcer (depending on the model as described above). After the induction of the ulcers the rats were then treated with ASEPA and fraction as stated in section 3.2.5 for 14 days after which the animals were sacrificed. The ulcer index and percentage inhibition (healing) were obtained as previously described in section 3.2.5.1.

3.2.6 Biochemical analysis

Estimation of catalase (CAT) and malondialdehyde (MDA).

The stomach from stress-induced ulcer were used for biochemical analysis. The excised stomach from the control and treated rats were weighed and chilled in ice cold saline. A stomach homogenate prepared in KCl (1.15 % w/v) was further utilized for biochemical analysis. Estimation of MDA and CAT levels in the stomach homogenate was determined according to the method described by Koracevic *et al* (2001) and Clairborne *et al* (1985) respectively.

Determination of catalse level

Stomach homogenate (0.3 ml) was gently mixed with 1.2 ml of 0.2M hydrogen peroxide and 3 ml of 0.01M phosphate buffer (7.0) and allowed to stand at room temperature for 5 minutes. From the reaction mixture was withdrawn 1 ml and blown into 2 ml of acetic acid reagent at 1 minute time interval. It was mixed and incubated in boiling water bath for 10 minutes, then cooled and the absorbance taken at 570 nm using spectrophotometer (Clairborne *et al.*, 1985).

Determination of Malondialdehyde (MDA) level

Assessment of oxidative stress was measured by determining the lipid peroxidation end product of MDA, using Thiobarbituric acid (TBA) (Koracevic *et al*, 2001). Tissue homogenate (2 ml) obtained as stated above was combined with 2 ml of 1 % TBA in 20 % NaOH and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. After cooling, the flocculate precipitate was removed by centrifugation at 1000 rpm for 10 minutes. The absorbance of the supernatant was measured at 532 nm against a blank that contains all the reagents minus the stomach homogenate. The MDA concentration of the sample was calculated using the extinction coefficient of 1.56x ($105M\text{-}1cm^{-1}$).

MDA concentration (M) =Abs/1.56 x 105

3.2.7 Effect of ASEPA and fractions on gastrointestinal transit

The method of Suchitra et al., (2003) was used. Forty albino rats of both sexes grouped into eight (n = 5) were used for this study. The rats were previously starved for 24 h with unrestricted access to drinking water and treated as follows:

Group 1: 10 ml/kg distilled water
Group 2: Injection Atropine (10 mg/kg, i.p)
Group 3: ASEPA 250 mg/kg
Group 4: ASEPA 500 mg/kg
Group 5: NF 250 mg/kg
Group 6: NF 500 mg/kg
Group 7: WF 250 mg/kg
Group 8: WF 500 mg/kg

Immediately after treatment, 0.5 ml of 5 % w/v of activated charcoal meal in tragacant solution (2 % w/v) was administered orally. The rats were allowed 15 minutes before being sacrificed with excess chloroform. After tying the intesting at the position of charcoal plug (in order to secure the position), the intestine from pylorus to ileocaecal junction was removed carefully and stretched out. The intestinal distance moved by the charcoal plug from the pylorus was measured and expressed as percentage of the total distance from the pylorus to the ileocaecal junction. The percentage inhibition was calculated based on that of the control.

3.2.8 Antimicrobial screening

Helicobacter pyrolus which is the enteric organism implicated in peptic ulcer disease could not be used in this screening test because of the difficulty in culturing the micro-organism. However, other gram negative entero-pathogenic and related microorganisms were employed. The bacteria used were: *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus aureus* and *Salmonella typhi* while the fungi were *Candida albican and Aspergillus niger*. They were obtained from the Department of

Pharmaceutical Microbiology Laboratory, School of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka, Nigeria. The organisms were maintained by weekly sub culturing and incubating at 37 ^{o}C (for bacteria) and 25 ^{o}C (for fungi). Twenty four-hour old cultures of the microorganisms were used in the screening and the agar disc diffusion method as described by Adeniyi *et al* (1996) was employed. Wells of 8 mm diameter were bored by using a standard cork borer 8 mm on seeded gelled agar dish (Mueller Hinton agar plates (MHA oxoid) England) containing 1.0 x 106 cfu/ml of the respective organism and varying concentrations of the extract and fractions were applied to the appropriately labeled wells. The plates were incubated at 37 ^{o}C for 24 h for bacteria and 48 h for fungi. The effects of the extract and fractions on the growth of the microorganisms were studied by observing the zones of inhibitions which were measured in millimeter (mm). The experiments were carried out in triplicates and the mean inhibition zone diameter obtained in each case.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) tests of ASEPA and fractions against the above listed micro-organisms were also carried out using liquid dilution method (Murray *et al.,* 1999). The tests were undertaken by setting up sterile test tubes of the extract solutions and test tube with only bacteria as a negative control. The antibiotic solution served as a positive control. A test tube filled with 0.5 ml of nutrient broth medium (NB) and 0.5 ml of the extract (or fraction) solution was added and homogenized. From the tube a 0.5 ml was transferred into a second tube. The serial dilution continued until the concentration of 1500 μg/ml of the extract (or fraction) was obtained. From the last tube 0.5 ml was removed and discarded so that each tube contained 0.5 ml. Into each tube was added 0.1 ml of bacterial suspension and 0.4 ml of NB making a total volume of 1 ml in each tube. After homogenization the mixtures were incubated at 37 °C for 24 h. Turbidity was

observed and compared with the positive control (NB + antibiotic) and a negative control (NB + bacteria). The lowest concentration showing no turbidity was considered as the MIC of the organism.

The minimum bactericidal concentration (MBC)

MBC values for the tested micro-organisms were determined from broth dilution minimum inhibitory concentration (MIC) tests by subculturing to agar plates that do not contain the test agent. MBC tests were undertaken by spreading a loopful evenly from the tube solution of 6000, 3000 and 1500 μg/ml over MBC media Muller Hinton agar solid. Media that had been subcultured were then incubated at 37 °C for 24 h. After 24 h, the lowest concentrations that did not show any growth of bacterial colonies on the solid media were considered as the MBC of the organism (Murray *et al.*, 1999)**.**

3.2.9 In vitro pharmacological studies

The effect of ASEPA and fractions on isolated tissue preparation was studied using segments of the ileum (2-3 cm long) suspended in 20 ml Tyrode solution in an organ bath. The composition of the Tyrode solution (mM/L) was NaCl-136.7, KCl-2.7, $CaCl_2$-1.8, $NaHCO_3$-11.9, $MgCl_2$-1.0, $Na2HPO_4$-0.4, and glucose-5.5. The solution was maintained at 37 ± 1 °C and aerated with air. The preparation was set up under a resting tension of 0.5 g (for guinea pig ileum) and 1 g (for rat and rabbit ileum) and allowed to equilibrate for 10 min. At the end of equilibration period, the extract and fractions were tested for spasmogenic or spasmolytic activity by adding increasing concentrations of the extract and fractions (mg/ml) to the bath preparations. The contact time for the activity was 30 seconds while the standard spasmogen acted for 20 seconds in a 3 minute time cycle. Responses were determined in triplicate and recorded using 4-channel recorder-17304 (Ugobasile Italy). The effects of ASEPA and fractions on submaximal responses to acetylcholine and histamine were obtained.

3.2.10. Histological study of the stomach.

Histological study of the stomach specimen from pyloric ligature and stress-induced ulcer models were carried out using the method described by Drury and Wallington (1980). The harvested stomach tissues which were preserved in 10 % formaldehyde solution were dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin wax. Sections (6 mm in thickness) of the tissues were prepared and stained with Haematoxylin and Eosin and subsequently examined under microscope. The photomicrographs were obtained.

3.2.11 Identification of the major compounds

Analytical high performance liquid chromatography (HPLC) was used for identification of the major compounds. Each of the dried extracts (ASEPA, NF, and WF) was reconstituted with 2 ml of HPLC grade methanol. The dissolved samples (100 µL) were each transferred into HPLC vials containing 500 µL of HPLC grade methanol. HPLC analysis was carried out on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, DionexSoftron GmbH, Germering, Germany). Detection was at 235, 254, 280 and 340 nm. The separation column (125 cm × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany) and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent**.**

3.2.12 Vaccum liquid chromatography (VLC) of NF

The VLC analysis of n-hexane fraction was carried out according to methods described by Cruz *et al.*, (2016). The n-hexane fraction (6 g) was mixed with 300 g silica gell (mesh 200-400), air-dried and eluted with 500 ml of various ratio of n-hexane: ethyl acetate mixture (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10). These resulted into 11

fractions. Thin layer chromatography (TLC) of the eleven fractions was carried out to confirm presence of compounds. The fractions were then allowed to air dry and weighed.

3.2.13 Anti-ulcer activity of the VLC fractions.

Fractions eluted with 8:2, 7:3, and 5:5 n-hexane: ethylacetate solvent combinations were found to contain some precipitate after drying. These eluents were labeled NX, NY and NZ respectively. Anti-ulcer activity investigation was carried out with these three fractions in mice. The 40 adult mice of both sexes weighing between 25 to 35 g were grouped into 8 (n=5) as follows:

Group 1: Control (distilled water 10 ml/kg)
Group 2: Cimetidine (150 mg /kg)
Group 3: NX 250 mg/kg
Group 4: NX 500 mg/kg
Group 5: NY 250 mg/kg
Group 6: NY 500 mg/kg
Group 7: NZ 250 mg/kg
Group 8: NZ 500 mg/kg

The mice were fasted for 24 h with access to water and treated with respective dose of the fraction as stated above. After 45 minutes, asprin (150 mg/kg) was administered orally and the animal sacrificed 4 h later by cervical dislocation. The stomach was opened along the greater curvature and washed with water. The degree of ulcer was recorded with the aid of hand lens using the ulcer grading method described in section 3.2.5.1.

3.2.14 Structural elucidation of compounds using GC-MS

The most active fraction of the three VLC fractions was subjected to gas column-mass spectrometry (GC-MS) analysis using Shimdzu (GCMS-QP2010plus) system Tokyo, Japan equipped with a AOC-20i auto-sampler according to the method described by Pripdeevech *et al* (2010). The columns used were an Rxi-sms capillary column (30m x 0.25 mm x 0.25 µm) (Belonite, PA, USA). The stationary phase was 5 % two phenyl, 95

% two methyl polysiloxanewhile and Helium was the carrier gas (0.7 ml/min). The injector temperature was 250 °C and the column temperature was maintained at 40 °C for 5 min and then programmed at 4 °C/min to 250 °C. The spectrometers were operated in electron ionization (EI) mode at 70 eV ionization energy; the scan range was 35–400 amu. The detector was set as fixed voltage at 1200V and the scan rate was 0.5 s per scan. The ionization source temperature was 250 °C. The identification of the major compounds was performed by comparing their mass spectra with the Wiley Registery of Mass spectra 8th edition library available in the instrument and confirmed by comparing with standards from national institute of standards and technology, mass spectra libraries (NIST).

3.2.15 Statistical analysis

The data were analyzed by statistical package for social sciences (SPSS version 20) using one way ANOVA, followed by post-hoc turkey's test for multiple comparisons. The data were expressed as mean ± Standard error of mean (SEM). Graphical representation was done using Microsoft excel 2010. The difference between mean were considered significant at $p<0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Yield of extract and fractions

The weight of milled *P. americana* seeds was 2 kg and the crude extract obtained from it was 305.1 g representing a percentage yield of approximately 15.3 % w/w**.** N-hexane yield was 170. 65 g representing a percentage yield of approximately 55.9 % w/w while water fraction yield was 90.85 g representing a percentage yield of approximately 29.7 % w/w. The weight of eluents from VLC analysis labeled NX, NY and NZ were 102 mg, 310 mg and 153 mg respectively. The percentage yield for NX, NY and NZ were approximately 1.7 % w/w, 5.2 % w/w and 2.6 % w/w respectively.

4.1.2 Acute toxicity study (LD_{50})

Following 24 h of oral administration of up to 5000 mg/kg dose of ASEPA, no lethality or any other sign of acute toxicity was seen or noted. The LD_{50} was therefore greater than 5000 mg/kg.

4.1.3 Phytochemical analysis

The qualitative phytochemical study of ASEPA and fractions revealed the presence of some bioactive substances which were mostly concentrated in ASEPA (Table 3). ASEPA contained alkaloids, saponins, flavonoids, tannins, terpenoids, cardiac glycosides and reducing sugars while NF and WF contain all the bioactive substances in ASEPA at lesser concentration. The quantitative analysis of ASEPA showed that reducing sugar had the highest concentration (14.2 %) followed by saponins (12.25 %) while flavonoid was the least with concentration of 0.49 % (Table 3).

Table 3 Result of the Phytochemical Screening

Sample	Alkaloid	Saponin	Tannin	Flavonoid	Steroid	Terpenoid	Cadiac glycosides	Reducing sugar
Constituents (%)	**4.7**	**12.25**	**3.8**	**0.49**	**-**	**4.7**	**11.6**	**14.2**
ASEPA	+	+++	+	+	_	+	++	+++
NF	+	++	+	+	_	+	+	+
WF	+	+	+	+	_	+	+	++

Key: +=trace or mildly present, ++=moderately present, +++= abundantly present
- =absent

4.1.4 Effect of the extract and fractions on ethanol-induced ulcer

The administration of 96 % v/v ethanol (p.o) produced ulceration in the control. ASEPA and fractions exhibited dose-dependent ulcer protective effect on ethanol-induced ulcer. At only 250 mg/kg of n-hexane fraction produced significant ($p<0.05$) reduction in ulcer index when compared to control. But at 500 mg/kg, oral treatment with ASEPA and fractions significantly ($p<0.05$) reduced the ulcer indexes in all the treated groups when compared to the control. The ulcer indexes of ASEPA (1.45 ± 0.13) and NF (1.17 ± 0.15) (500 mg/kg) treated groups were lower than that of cimetidine (150 mg/kg) group (2.30 ± 0.35) (Table 4)

4.1.5 Effect of the extract and fractions on aspirin-induced ulcer

The ASEPA and fractions conferred a dose-dependent ulcer protective effect on aspirin induced ulcer model. This was shown by the significant ($p<0.05$) reduction in ulcer indexes in the 500 mg/kg treated groups when compared to the control. NF 500 mg/kg caused the best anti-ulcer effect (0.94± 0.32) which was comparable with that of cimetidine (0.94 ± 0.22) (Table 4).

4.1.6 Effect of the extract and fractions on stress-induced ulcer

Stress resulted in increase in ulceration in the control. ASEPA and fraction exhibited a dose-dependent ulcer protective effect against stress-induced ulcer. At 250 mg/kg only ASEPA and n-hexane fraction caused significant ($p<0.05$) reduction in ulcer index in the treated groups, whereas at 500 mg/kg ASEPA and all the fractions caused significant ($p<0.05$) reduction in ulcer indexes of all the treated groups (Table 4). At 500 mg/kg, the ulcer indexes for NF treated group (2.01 ± 0.20) and ASEPA treated group (1.66 ± 0.19) were lower than that of cimetidine (2.06 ± 0.05) (Table 4).

4.1.7 Effect of extract and fractions on pyloric-ligature induced ulcer

The extract and fraction also showed a dose-dependent reduction in the pyloric-ligature induced ulcer, which was shown by the significant ($p<0.05$) decrease in ulcer index by the

500 mg/kg when compared to the control. At the dose of 250 mg/kg, only n-hexane treated group showed significant ($p<0.05$) reduction in ulcer index when compared to control (Table 4).

4.1.8 Healing effects of ASEPA and fractions on different ulcer models

ASEPA and fractions conferred a dose-dependent healing and reduction in ulcer index in all the three ulcer models (ethanol, asprin and stress). At 500 mg/kg ASEPA and fractions showed significant ($p<0.05$) reduction in ulcer index in all the ulcer models when compared to control. For ethanol induced ulcers, ASEPA, NF, and WF (500 mg/kg) conferred better healing effect than cimetidine (150 mg/kg) (Table 5).

4.1.9 Effect of on gastric acid volume, pH, free acidity and total acidity

Effect on gastric acid volume

There was significant ($p<0.05$) reduction in gastric acid volume in all the treated groups (except WF 250 mg/kg) when compared to the control. At 500 mg/kg, the gastric acid volume for ASEPA (0.21 ± 0.03) and NF (0.27 ± 0.02) were lower than that of cimetidine (0.29 ± 0.01) (Table 6).

Effect on gastric acid pH

The extract and fractions caused a dose-dependent increase in pH. At 500 mg/kg there was a significant ($p<0.05$) increase in pH in all the treated groups when compared to control. The gastric acid pH of cimetidine treated group was however higher than that of the extract and fraction treated groups (Table 6).

Effect on free acidity

Significant ($p<0.05$) reduction in free acidity was evident in the extract (at 500 mg/kg) and fractions (at 250 and 500 mg/kg) treated groups. Cimetidine (150 mg/kg) however exhibited lowest free acidity (Table 6). This effect was dose-depndent.

Table 4 Ulcer Indexes (UI) for the Ulcer-Protective study

Treatment	Dose/kg	Ethanol 1ml/200 mg	Asprin 150 mg/kg	Stress induced.	Pyloric ligature
Dist.water	**10 ml**	3.38 ± 0.17	2.65 ± 0.19	3.63 ± .16	4.56 ± 0.16
Cimetidine	**150 mg**	2.30 ± 0.35*	0.94 ± 0.22*	2.06 ± 0.05*	2.76 ± 0.16*
ASEPA	**250 mg**	2.80±0.36	2.45 ± 0.21	2.13 ± 0.51*	4.21 ± 0.20
	500 mg	1.45 ± 0.13*	0.95 ± 0.19*	1.66 ± 0.19*	2.91 ± 0.12*
NF	**250 mg**	2.41 ± 0.36*	2.35 ± 0.25	2.24 ± 0.59*	3.12 ± 0.17*
	500 mg	1.17 ± 0.15*[a]	0.94± 0.32*	2.01 ± 0.20*	2.28 ± 0.14*
WF	**250 mg**	2.68 ± 0.37	2.05 ± 0.20	2.95 ± 0.48	4.38 ± 0.15
	500 mg	1.60 ± 0.20*	1.55 ± 0.17*	2.47 ± 0.17*	2.98 ± 0.10*

Values are represented as mean ± standard error of mean (n=5). *p<0.05: Statistically significantly different from the control group. Key: ASEPA=aqueous seed extract of *P. americana*, NF=n-hexane fraction, WF=water fraction.

Table 5 Ulcer indexes (UI) and percentage healing effect (% H) of ASEPA and fractions

Treatment	Dose/kg	Ethanol 1ml/200 mg		Asprin 150 mg/kg.		Stress induced.	
		UI	**%H**	**UI**	**%H**	**UI**	**%H**
Dist.water	**10 ml**	3.28 ± 0.14	**0**	2.55 ±0.16	**0**	3.53 ± .13	**0**
Cimetidine	**150 mg**	2.20 ± 0.32*	**34**	0.84 ± 0.19*	**52**	1.96 ±0.02*	**44**
ASEPA	**250 mg**	2.70±0.33	**18**	2.35 ±0.18	**7**	2.03 ±0.48*	**42**
	500 mg	1.35 ± 0.10*	**58**	0.85 ±0.16*	**66**	1.56 ±0.16*	**56**
NF	**250 mg**	2.31 ± 0.33*	**30**	2.25 ± 0.22	**11**	2.14 ±0.56*	**39**
	500 mg	1.17 ± 0.12*[a]	**64**	0.94± 0.29*	**63**	1.91 ±0.17*	**46**
WF	**250 mg**	2.58 ± 0.34	**21**	1.95 ± 0.17	**24**	2.85 ± 0.45	**19**
	500 mg	1.50 ± 0.17*	**54**	1.45 ± 0.14*	**43**	2.37 ±0.15*	**33**

Values are represented as mean ± standard error of mean (n=5).
***p<0.05:Statistically significantly different from the control group.** Key: ASEPA= aqueous seed extract of *P. americana*, NF=n-hexane fraction WF=water fraction

Effect on total acidity

Reduction in total acidity by ASEPA and NF at 500 mg/kg was significant ($p<0.05$) when compared to control (Table 6).

4.1.10 Effect of the extract and fractions on biochemical parameters

Effect on catalase (CAT) level

The extract and fractions exhibited a dose-dependent increase in CAT level. At 500 mg/kg ASEPA, NF and WF exhibited a significant ($p<0.05$) increase in CAT level when compared to control (Table 7).

The effect on malondialdehyde level (MDA).

ASEPA and fraction showed a dose-dependent reduction on MDA level. NF and WF (500 mg/kg) produced significant ($p<0.05$) reduction in MDA level when compared to control (Table 7).

4.1.11 Effect of extract and fractions on gastro-intestinal transit

The reduction in GIT motility shown by ASEPA and fractions was dose-dependent. ASEPA and WF (500 mg/kg) exhibited a significant ($p<0.05$) reduction in distance travelled by charcoal meal when compared to control. NF did not significantly ($p<0.05$) reduce the GIT motility at 250 and 500 mg/kg (Table 8).

4.1.12 Anti-microbial effect of extract and fractions

The ASEPA and fractions exhibited concentration-dependent anti-microbial activity against all the tested organisms; *S. aureus, B. subtilus, E.coli, P. aeruginosa, S.typhi, Candida albican,* and *A. niger*, ranging from 25 to 200 mg/ml. ASEPA and the fractions were more effective on the fungi than bacteria. At 200 mg/ml ASEPA, NF and WF demonstrated the highest inhibition zones of 37, 36 and 47 mm against S.*aureus, E.coli and E.coli* respectively. ASEPA had the highest MIC (44.2 mg/ml) and the lowest MIC 20.1 mg/ml) against *S. aureus* and *A. niger* respectively. For MBC, ASEPA has the

Table 6 Effect of extract and fractions on gastric volume, pH, total and free acidity.

Treatment	Dose mg/kg	Gastric content(ml)	pH	Free acidity	Total acidity
Distilled water 10 ml	10 ml	0.98 ± 0.06	2.74 ± 0.07	6.33 ± 0.03	0.68 ± 0.12
Cimetidine	150	0.29 ± 0.01*	4.41 ± 0.13*	3.26 ± 0.08*	0.28 ± 0.01*
ASEPA	250	0.32 ± 0.01*	2.65 ± 0.21	5.81 ± 0.29	0.65 ± 0.02
	500	0.21 ± 0.03*	3.28 ± 0.03*	4.86 ± 0.27*	0.35 ± 0.06*
NF	250	0.27 ± 0.02*	2.40 ± 0.09	5.06 ± 0.05*	0.41 ± 0.03*
	500	0.18 ± 0.02*	3.52 ± 0.17*	4.63 ± 0.03*	0.43 ± 0.03*
WF	250	0.89 ± 0.02	2.60 ± 0.11	4.80 ± 0.20*	0.60 ± 0.07
	500	0.50 ± 0.03*	3.31 ± 0.13*	4.00 ± 0.03*	0.56 ± 0.01

Values are represented as mean ± standard error of mean (n=5). *p<0.05: Statistically significantly different from the control group. Key: ASEPA= aqueous seed extract of *P.americana*, NF=n-hexane fraction WF=water fraction

Table 7 Effect of ASEPA and Fractions on Biochemical parameters

Treatment	Dose mg/kg	CAT level (u/l)	MDA level (nm/mg)
Distilled water	10 ml	25.95 ± 2.69	1.02 ± 0.01
Cimetidine	150	70.20 ± 0.53*	0.54 ± 0.03*
ASEPA	250	28.45 ± 0.33	0.88 ± 0.01
	500	63.86 ± 0.49*	0.60 ± 0.01*
NF	250	29.42 ± 2.00	0.78 ± 0.01
	500	65.62 ± 2.54*	0.52 ± 0.01*
WF	250	26.82 ± 0.70	0.95 ± 0.03
	500	54.19 ± 1.89*	0.87 ± 0.02

Values are represented as mean ± standard error of mean (n=5). P<0.05: Statistically significantly different from the control group. Key: ASEPA= aqueous seed extract of *P.americana*, NF=n-hexane fraction WF=water fraction, (MDA)=malondialdehyde, CAT= catalase

Table 8 Effect of ASEPA and Fractions on Gastrointestinal Transit

Treatment	Dose (mg/kg)	Distance travelled by Charcoal meal (cm)	Inhibition of GIT transit (%)
Distilled water	10 ml	93.60	0
Atropine	10	19.40	79.27
ASEPA	250	72.20	22.86
	500	61.20	34.62
NF	250	81.40	13.03
	500	69.80	25.43
WF	250	67.40	27.99
	500	49.20	47.44

Key: ASEPA= Aqueous seed extract of *P.americana*, NF=n-hexane fraction WF=water fraction**.** Distance travelled by charcoal meal in control = 93.60 cm. Percentage inhibition was calculated with respect to that of control.

highest MBC value (52.1 mg/ml) and the lowest MBC (32.5 mg/ml) against *B. subtilus and C. albican* respectively. In the same way NF exhibited highest MIC value (32.5 mg/ml) against *S. aureus* and *P. aeruginosa* and lowest MIC value (20.3 mg/ml) against *S.typhi*. NF exhibited the highest MBC value (41.7 mg/ml) and lowest (30.1 mg/ml) against *S. aureus and S.typhi* respectively. WF exhibited the highest MIC value (28.4 mg/ml) and the lowest (18.4 mg/ml) against *Candida albican* and *S.typhi* respectively. WF exhibited the highest MIC value (28.4 mg/ml) and the lowest (18.4 mg/ml) against *Candida albican* and *S.typhi* respectively. WF exhibited the highest MBC value (35.4 mg/ml) against *E.coli*, and the lowest (25.5 mg/ml) against *S.typhi* (Table 9 and 10). WF seems to have better anti-microbial effect considering its low MIC and MBC values against most of the tested microorganisms.

4.1.13 In vitro Pharmacological Studies

Effects of the extract and fractions on isolated guinea pig ileum

The ASEPA, NF and WF caused non-dose dependent spasmolytic responses on the isolated guinea pig ileum (Figures 3, 4 and Appendix 2). The initial contractile response elicited by histamine (2ug) was partly reduced by NF (5 mg) and WF (5 mg) (Figure 3 and Appendix 2). The contractile response to acetylcholine (4 ug) was reduced by ASEPA (Figure 4 and Appendix 2). Likewise, the initial response to acetylcholine (0.2 ug) was reduced by NF (5 mg) and WF (5 mg) (Figure 5 and Appendix 2)

4.1.14 Histological effects of extract and fractions

The stomach of pyloric ligated rats exhibited a marked area of necrosis and discontinuity in the mucosal epithelium of the control rats (Figure 6A). The photomicrograph of the group treated with 500 mg/kg ASEPA (6B) also showed area of apical necrosis of mucosal epithelium as well as sub-mucosal edema (ED). Administration of n-hexane and water fraction (500 mg/kg) for two weeks before the pyloric ligation was cytoprotective.

Hence, the photomicrograph of the stomach of NF (Figure 6C) and WF (Figure 6D) treated rats displayed normal gastric mucosa (M), normal submucosa (SM)) and normal muscularis mucosa (MM)**.** The stomachs of the rats of stress- induced ulcer revealed, sub mucosal inflammation in the negative control rats (Figure 7A). Two weeks oral daily administration of ASEPA, NF and WF (500 mg/kg) before the induction of stress resulted in normal gastric mucosa (M), normal sub mucosa (SM)) and muscularis mucosa (MM) of their stomachs (Figures 7B, 7C, 7D respectively).

4.1.15 High pressure liquid chromatography (HPLC) of extract and fractions

HPLC chromatogram of ASEPA revealed 9 peaks which represents nine compounds with the most prominent being peak number 9 (Figure 8A) with the corresponding UV lamda max of 262.5nm at 235 wavelength suggesting Absciscic acid (Figure 8B).

There were 11 peaks in NF representing eleven compounds with the most prominent being peak number 9 (Figure 9A) with the corresponding UV lamda max of 262.8 nm at 235 wave length suggesting Absciscic (Figure 9B)

For WF there were 15 Peaks representing fifteen compounds with the most prominent being peak number 12 (Figure 10A) with the corresponding UV lamda max of 262.4 nm at 235 wave length suggesting Absciscic acid (Figure 10B). Hence, Absciscic acid was found to be the major compound present in the aqueous crude extract and fractions of *P. americana* seeds.

4.1.16 Anti ulcer activity of the three VLC fractions

All the VLC fractions significantly ($p<0.05$) reduced the ulcer indexes when compared to the control (distilled water 10 ml/kg). However, the ulcer indexes in the VLC treated groups were significantly ($p<0.05$) higher than that of cimetidin (150 mg/kg). Among the fractions, the percentage ulcer inhibition produced by NY at 250 mg/kg (42.31 %) and 500 mg/kg (48.38%) were higher than that of other fractions (Table 11).

Table 9 Anti-bacterial Effect of Extract and Fractions

Test organism	Concentration (mg/ml)					Ciprofloxacin
	200	**100**	**50**	**25**	**12.5**	50ug/ml
	Inhibition zone diameter (mm) for ASEPA					
Staph aureus	34	20	15	0	0	30
B. subtilis	27	18	13	0	0	29
P. aeruginosa	30	19	15	0	0	21
E. coli	35	28	19	0	0	36
Sal. typhi	37	26	19	6	0	30
C. albicans	29	20	11	4	0	21
A.niger	30	26	14	2	0	28
	Inhibition zone diameter (mm) for NF					
Staph aureus	29	19	10	0	0	30
B. subtilis	20	11	6	0	0	18
P. aeruginosa	23	10	5	0	0	25
E. coli	36	21	12	4	0	19
Sal. typhi	31	18	9	4	0	28
C. albicans	21	15	9	0	0	12
A.niger	27	12	7	2	0	15
	Inhibition zone diameter (mm) for WF					
Staph aureus	28	15	10	7	0	30
B. subtilis	35	16	9	0	0	32
P. aeruginosa	40	20	12	9	0	19
E. coli	47	25	16	11	0	38
Sal. typhi	29	15	11	6	0	18
C. albicans	34	21	10	0	0	18
A.niger	37	20	12	8	0	25

Table 10 MIC and MBC (mg/ml) of ASEPA and Fractions against the test Bacterial species

Samples	1		2		3		4		5		6		7	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ciproxin	39.5	50.8	40.2	50.2	20.8	30.6	19.8	30.4	21.6	28.2	25.9	28.1	19.8	24.7
ASEPA	44.2	51.2	40.7	52.1	35.2	37.9	40.2	49.8	22.4	32.5	22.9	32.5	20.1	32.6
NF	32.5	41.7	24.0	35.9	21.3	30.5	32.5	33.5	20.3	30.1	30.7	31.2	22.3	30.4
WF	21.9	32.2	27.3	29.8	21.2	35.4	20.3	35.0	18.4	25.5	28.4	30.3	20.4	25.6

Key: 1 = *S. aureus, 2= B. subtilus, 3= E.coli, 4= P. aeruginosa, 5= S.typhi, 6= Candida albican,* and 7= *A. niger*, MIC=minimum inhibitory concentration, MBC= minimum bactericidal concentration

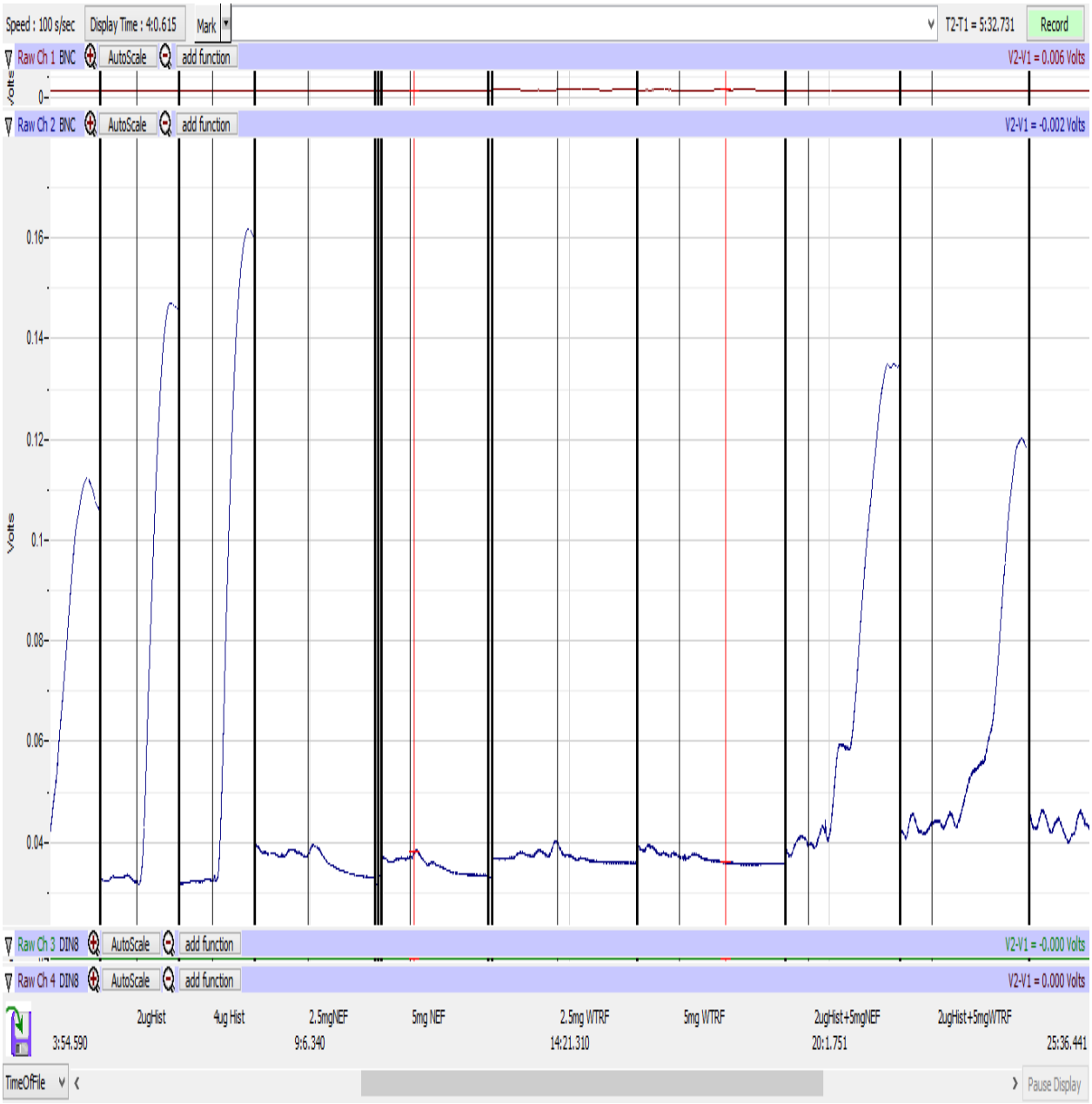

Figure 3. Non-dose dependent Effects of NF and WF and their Effects on Histamine-induced contraction on Guinea Pig ileum.
Key: NF=n-hexane fraction WF=water fraction

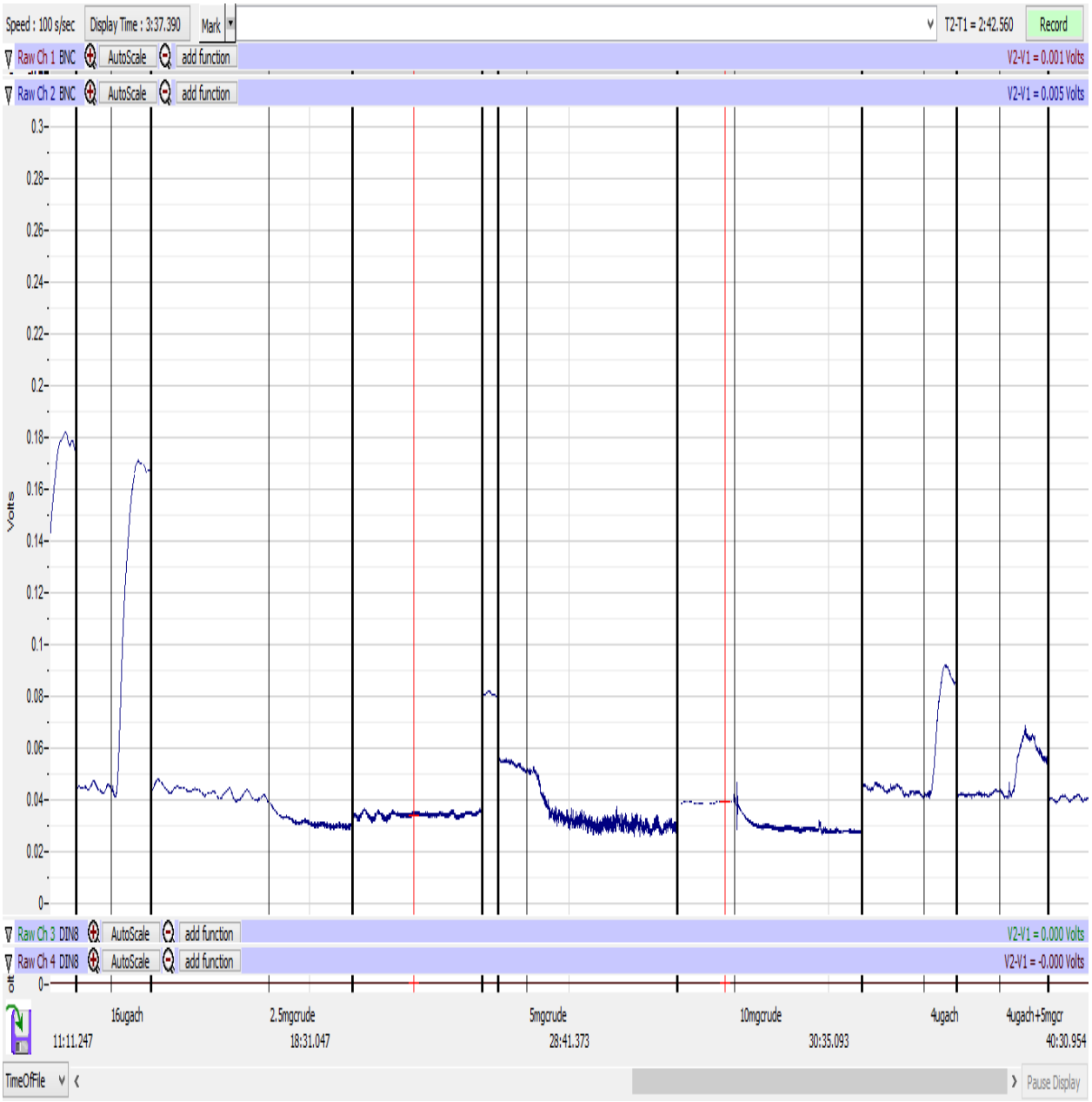

Figure 4. Non-dose dependent Effect of ASEPA and the Effect on Acetylcholine induced contraction on Guinea Pig ileum. Key: crude = ASEPA and ach= Acetyl choline

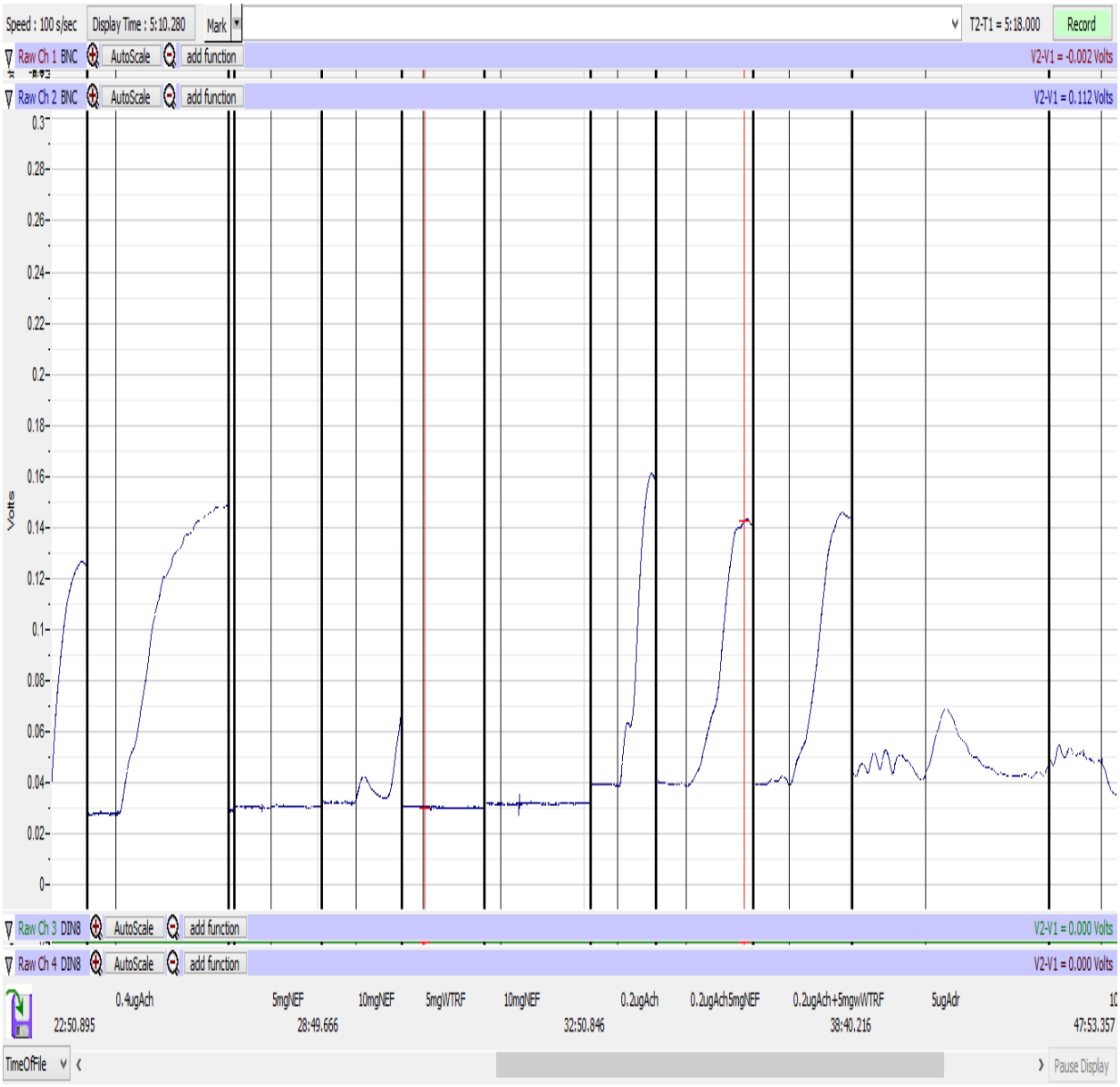

Figure 5. **Effect of NF and WF on Acetylcholine induced Contraction on Guinea Pig ileum.** Key**:** NF=n-hexane fraction, WF=water fraction, ach =Acetylcholine

4.1.17 GC-MS analysis of n-hexane fraction

The interpretation of the GC-MS analysis was conducted using the database of National Institute Standard and Technology (NIST) by comparing the unknown component with the spectrum of the known components stored in the library. The molecular weight and the structure of the compounds in the test materials were ascertained. The identification of the phytoconstituents was based on retention time, molecular weight, molecular formula and peak percentage. The GC-MS Spectra of the phytochemical constituents from n-hexane fraction (VLC NY fraction) revealed 24 compounds. Decane was the most abundant (9.99% of total oil composition) with retention time of 6.84 minutes while the least was 2,4 dimethyl heptane (1.57 % of total oil composition) with retention time of 4.6 minutes. All the compounds were saturated fatty acids (alkanes) (Figure 12, Table 12 and Appendix 3).

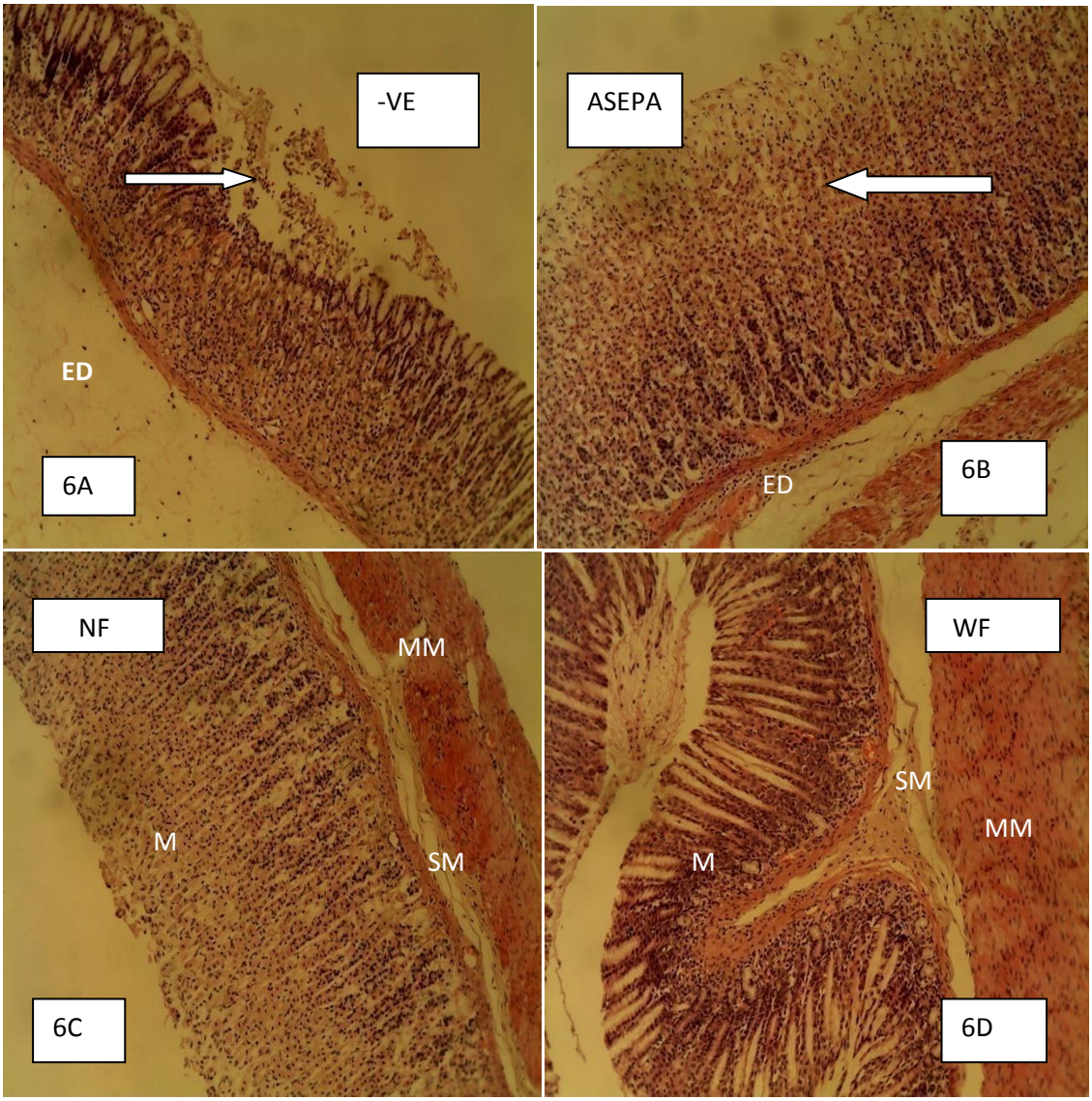

Figure 6. Photomicrographs of stomach of pyloric-ligated rats

The mucosal epithelium of the control (6A) with marked area of necrosis and discontinuity (white arrow), ASEPA treated group epithelium (6B) with area of apical necrosis of mucosal epithelium (white arrow) with edema of sub mucosa (ED). There was normal gastric mucosa (M), submucosa (SM)) and muscularis mucosa (MM) in NF and WF treated groups (Figure 6C and 6D)**.** Key: ASEPA= aqueous seed extract of *P.americana*, NF=n-hexane fraction, WF=water fraction

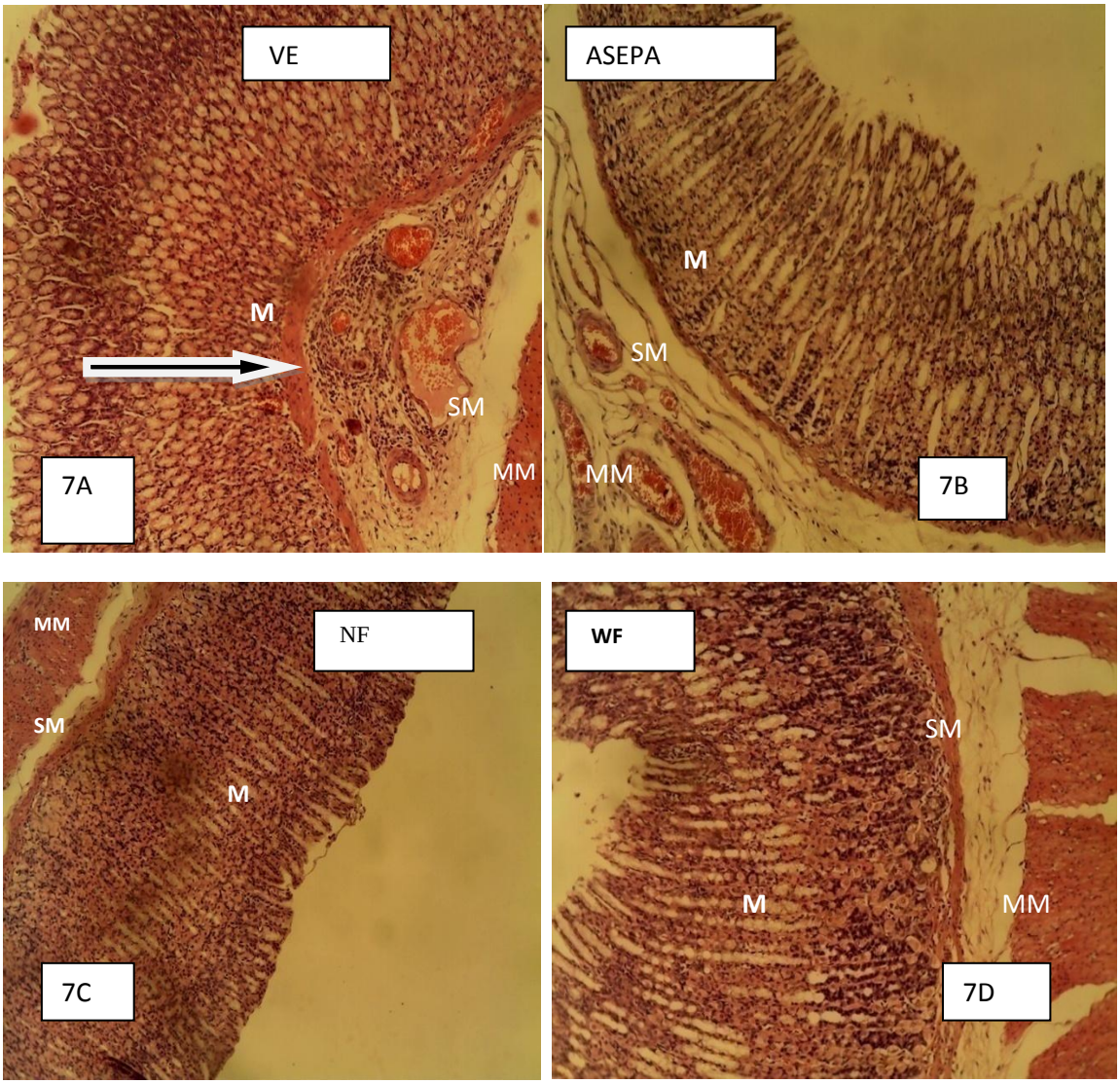

Figure 7. Photomicrograph of stomach of rats from stress induced ulcer

Black arrow shows submucosal inflammation in the negative control (7A**)** and apparently normal gastric mucosa (M), submucosa (SM)) and muscularis mucosa (MM) in ASEPA, NF and WF (Figures 7B, 7C and 7D respectively)**.** Key: ASEPA= aqueous seed extract of *P.americana*, NF=n-hexane fraction, WF=water fraction

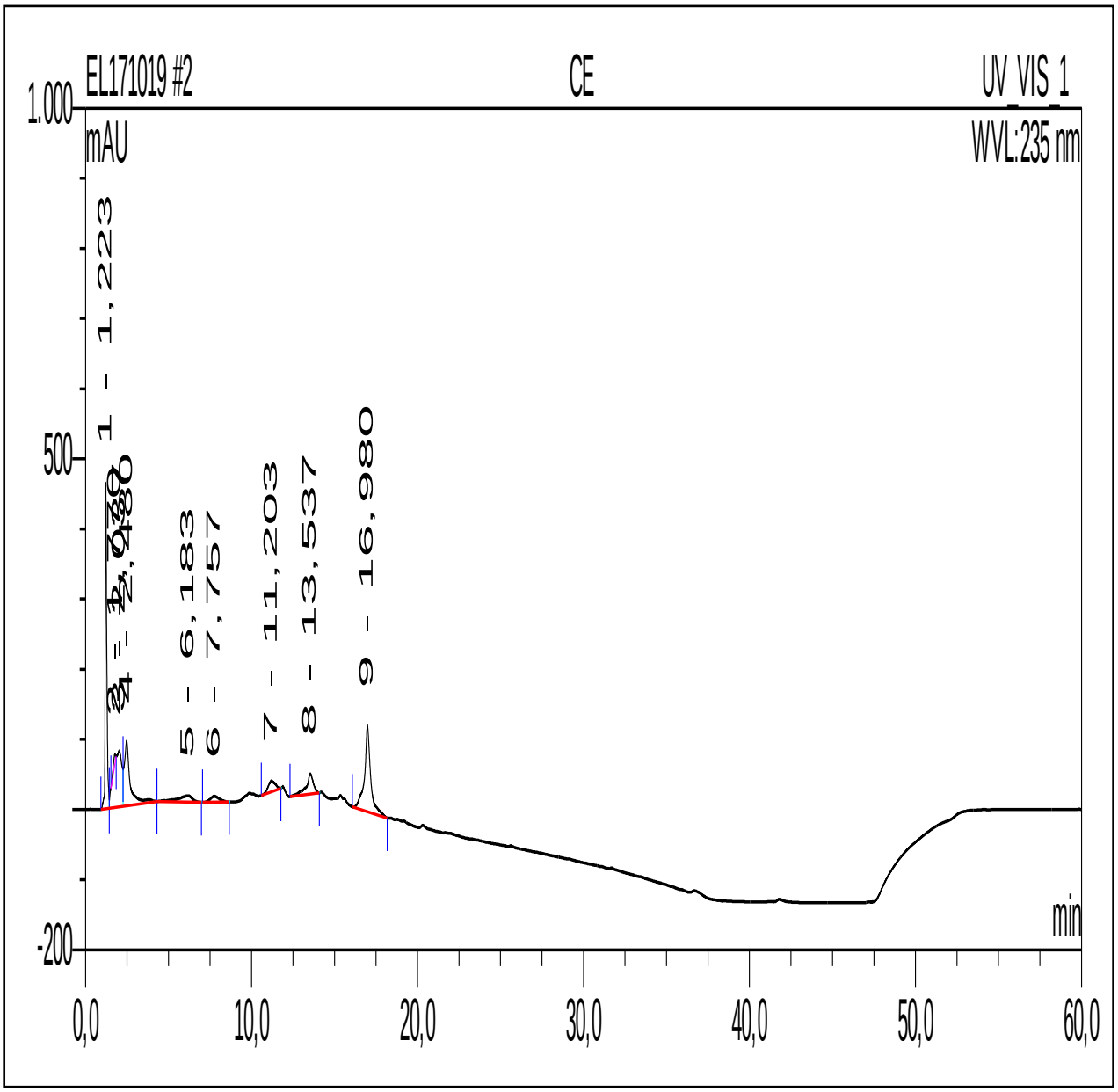

Figure 8A. HPLC Chromatogram of ASEPA showing Peak number 9 as the most prominent Peak. Key: CE=Crude extract (ASEPA**).**

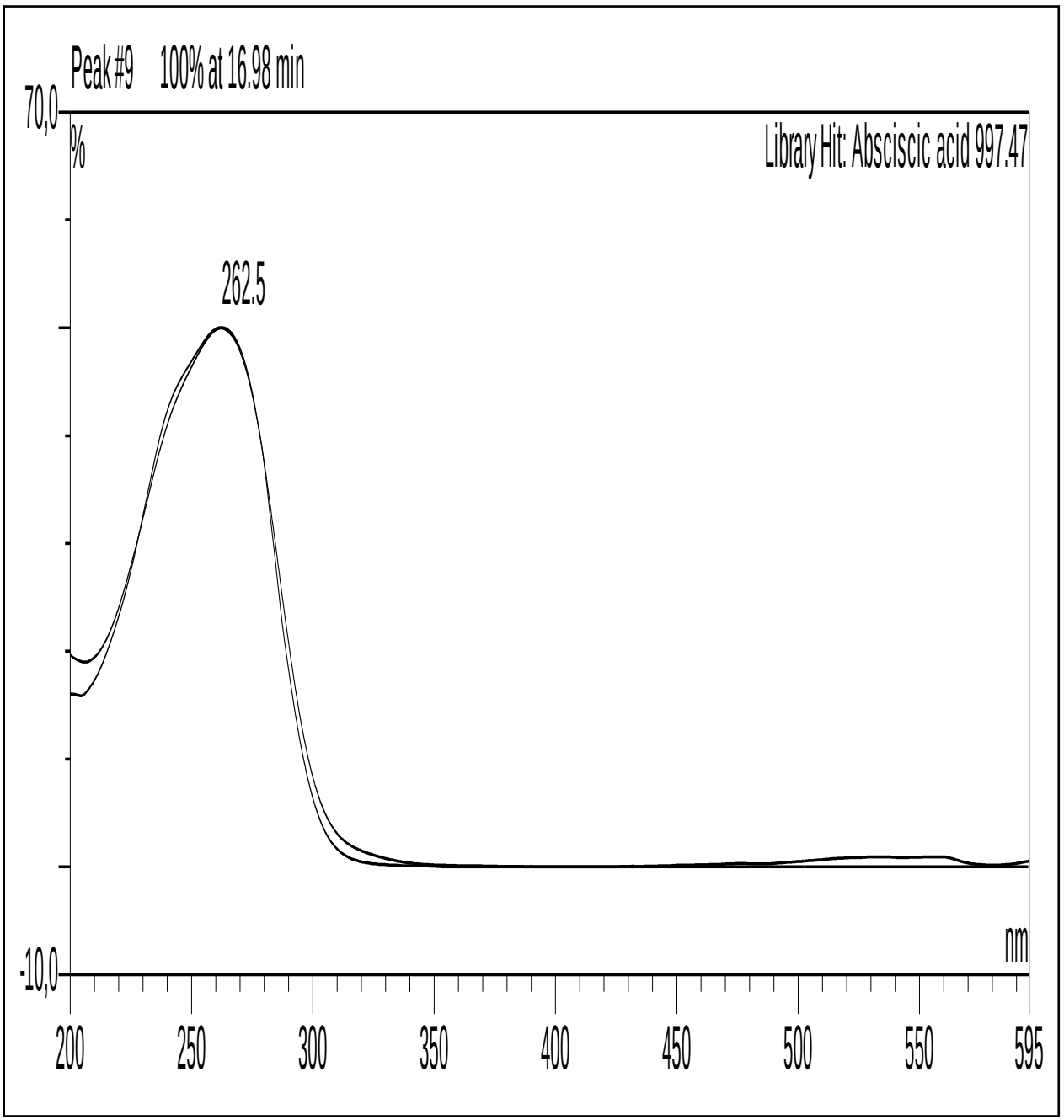

Figure 8B. UV spectrum of peak 9 of HPLC chromatogram of ASEPA. UV lamda max for peak 9 was 262.5nm suggesting Absciscic acid at 235 wavelength. Key: CE=Crude extract (ASEPA)

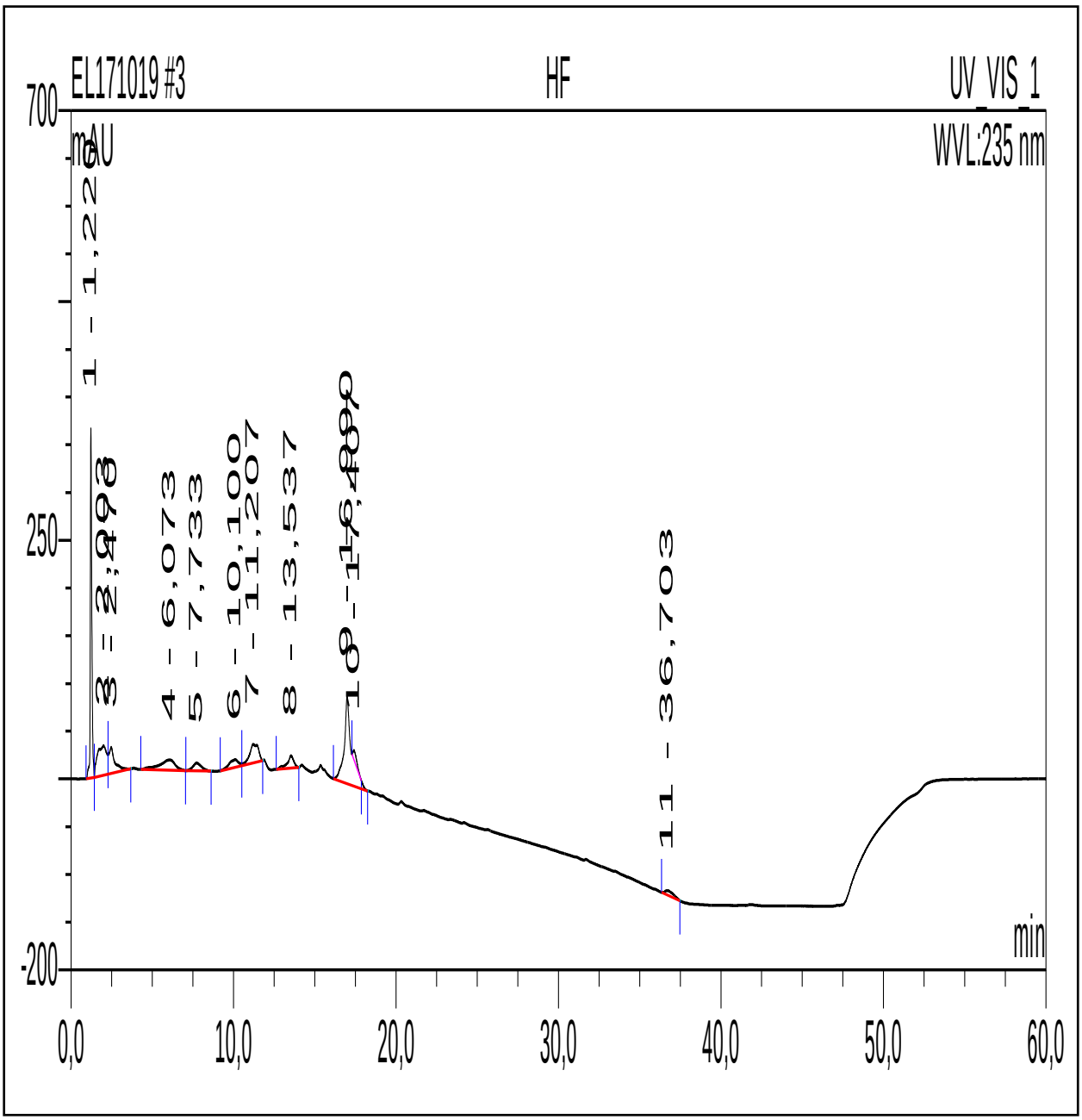

Figure 9A. HPLC Chromatogram of NF showing Peak 9 as the most prominent Peak. Key: NF= n-hexane fraction (HF)

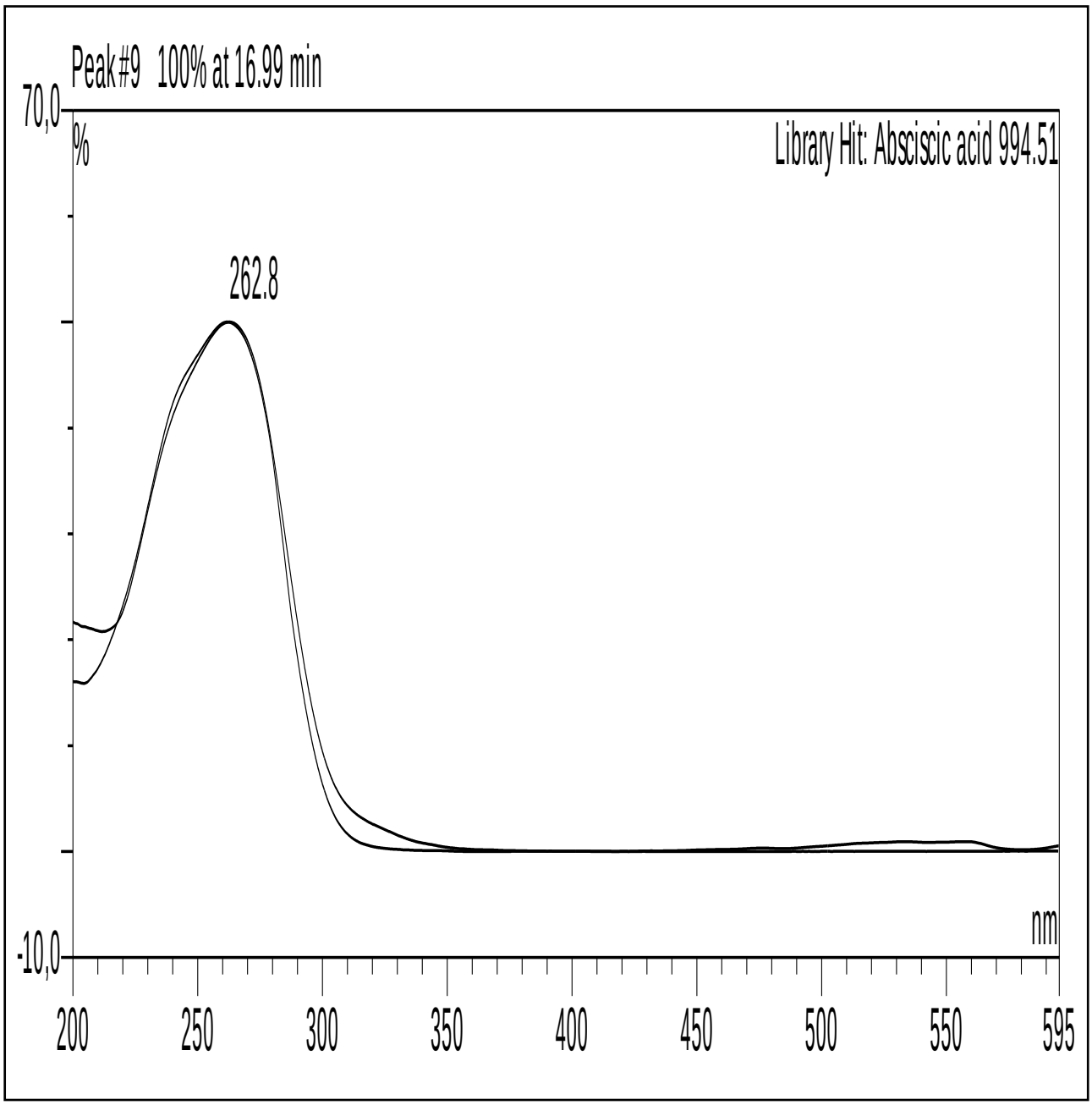

Figure 9B. UV spectrum of Peak 9 for NF fraction. UV lamda max for peak 9 was 262.8nm suggesting Absciscic acid at 235 wavelength. Key: HF=hexane fraction

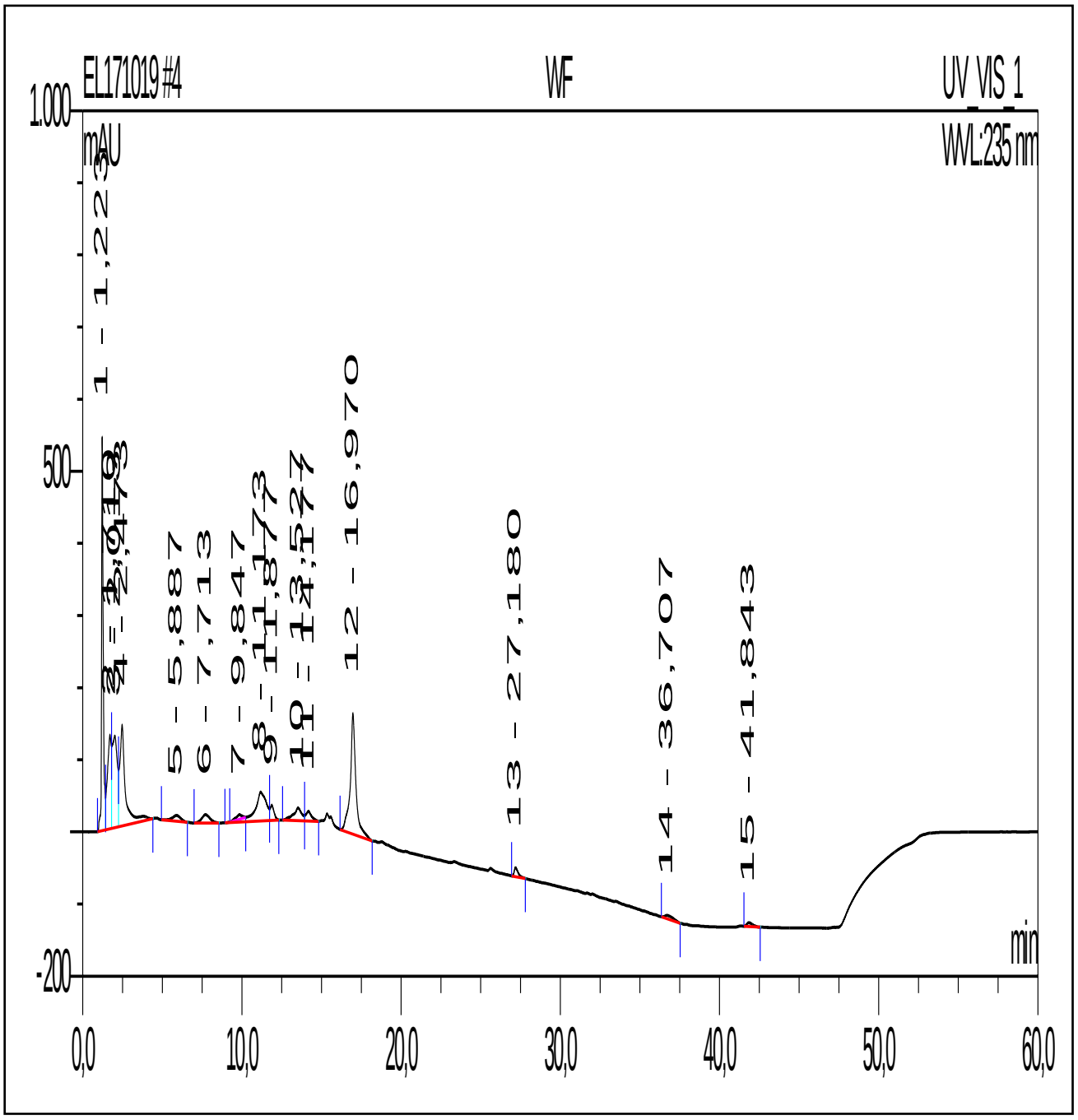

Figure10A. HPLC Chromatogram of WF showing Peak 12 as the most prominent Peak. Key: WF=water fraction

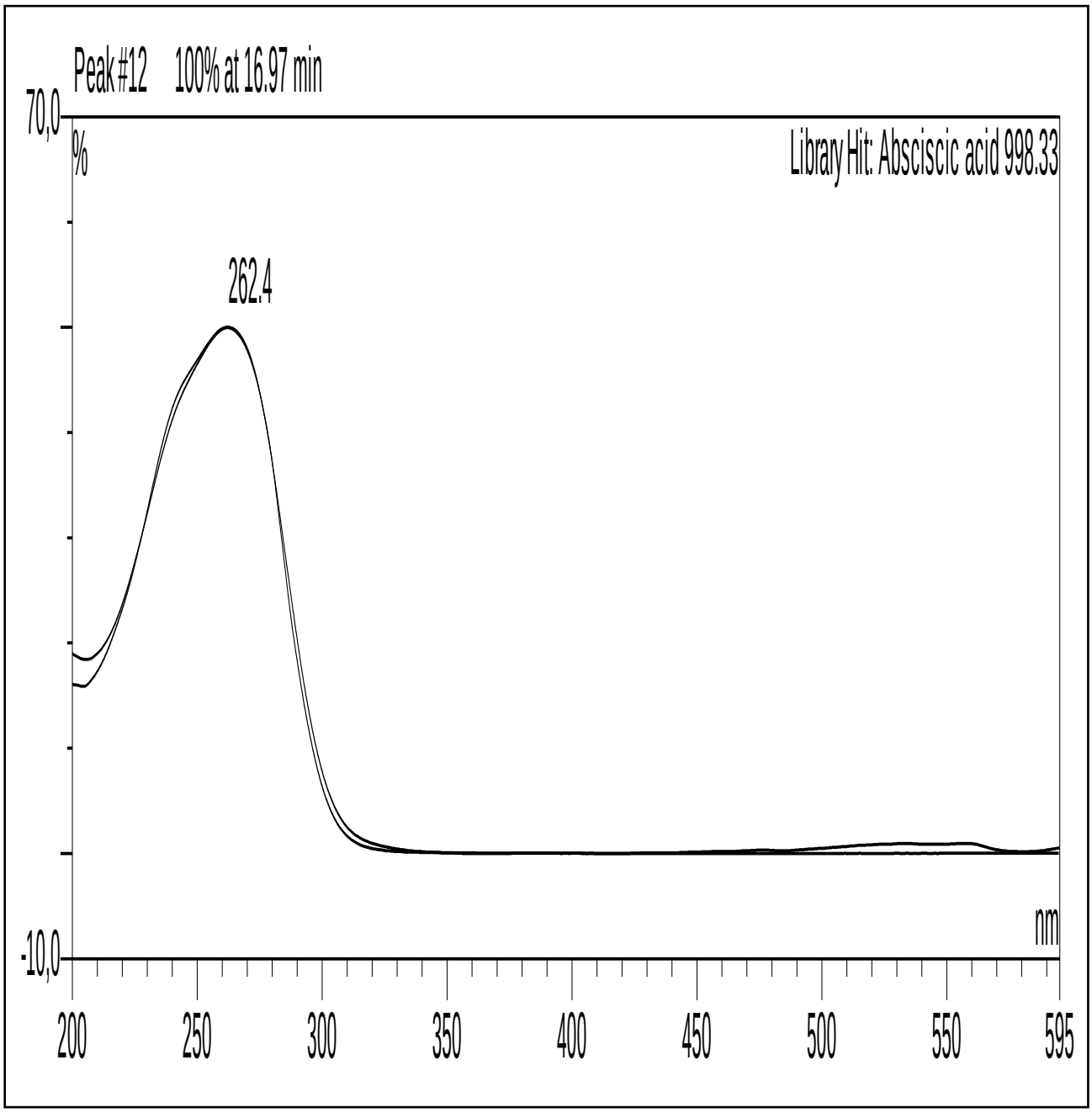

Figure 10B. UV spectrum of peak 12 for WF. UV lamda max for peak 12 was 262.4nm suggesting Absciscic acid at 235 wave length. Key: WF=water fraction.

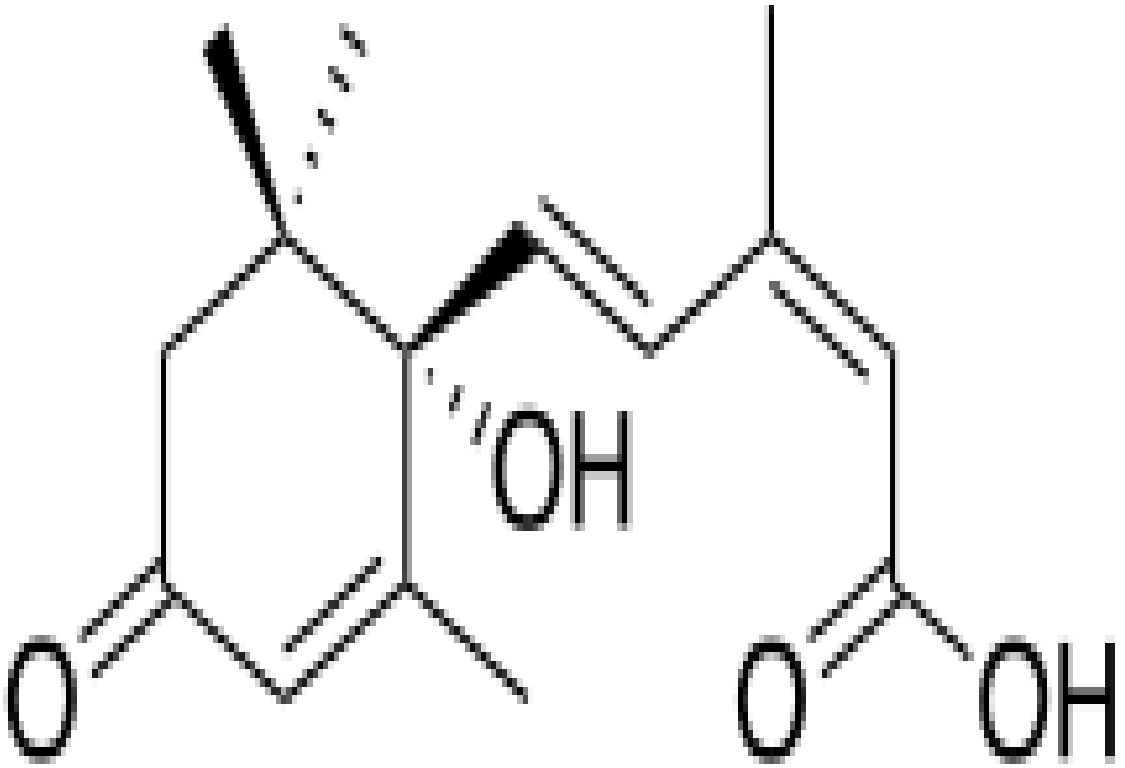

Figure 11.Chemical Structure of Abscisic Acid (**ABA)**
(National Center for Biotechnology Information. 16 September 2004)

Table 11 Anti ulcer Activity of the VLC Fractions

Treatment	Dose/kg	Ulcer index(UI)	Inhibition (%)
Distilled water	10 ml	5.20 ± 0.11	0.00
Cimetidine	150	0.64 ± 0.29*	87.73
VLC NX	250	3.42 ± 0.09*[a]	34.23
	500	3.36 ± 0.20*[a]	35.42
VLC NY	250	3.00 ± 0.03*[a]	42.31
	500	2.68 ± 0.16*[a]	48.38
VLC NZ	250	3.80 ± 0.07*[a]	26.92
	500	3.89 ± 0.09*[a]	25.19

Values are presented as mean ± Standard error of mean (SEM) n =5. *p<0.05: Statistically significantly different from control group. [a]p<0.05: Statistically significantly different from Cimetidine (150 mg/kg) group.

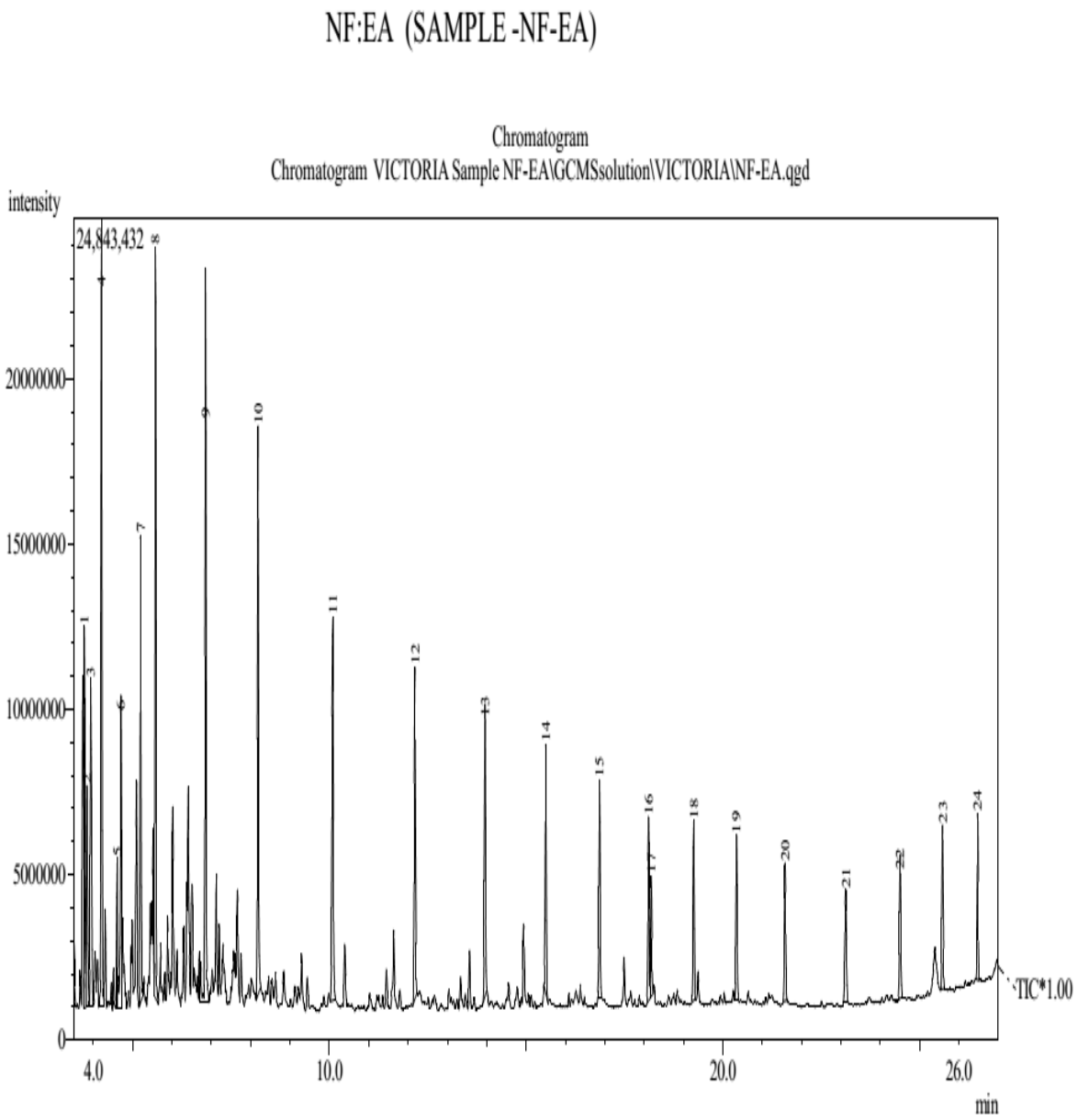

Figure 12. Chromotogram of GC-MS Analysis of n-hexane Fraction

Table 12 24 Compounds from GC-MS analysis

S/n	Name of compound	Retention time(min)	% Peak area	Molecular weight
1	Methyl benzene	3.76	4.12	92.14
2	3 methyl heptane	3.83	2.77	114.23
3	p-dimethyl cyclohexane	3.93	4.93	112.22
4	Octane	4.2	9.38	114.23
5	2, 6-dimethyl heptanes	4.6	1.57	128.26
6	1 ethyl 3 methyl cyclopentane	4.69	4.03	112.22
7	1,3-cyclopentadine	5.2	5.95	106.17
8	Nonane	5.58	8.38	128.26
9	Decane	6.84	9.99	142.29
10	Decane	8.18	7.39	142.29
11	Decane	10.08	6.42	142.29
12	Tridecane	12.18	4.92	184.37
13	Tridecane	13.96	4.05	184.37
14	Tridecane	15.5	3.35	184.37
15	Hexadecane	16.87	2.93	226.45
16	Hexadecane-	18.10	2.47	226.45
17	2, 6, 11-trimethyl dodecane	18.18	1.67	212.42
18	Hexadecane-	19.26	2.28	226.45
19	Hexadecane	20.34	2.34	226.45
20	Hexadecane	21.58	2.24	226.45
21	Hexadecane	23.13	2.20	226.45
22	Hexadecane	24.5	2.25	226.45
23	2-methyl nonadecane	25.58	2.25	282.56
24	2, 4-dimethyl eicosane	26.478	2.13	310.61

4.2 DISCUSSION

The etiology of peptic ulcer is assumed in most cases, yet it is generally accepted that it results from an imbalance between aggressive factors and the endogenous defense mechanisms (https://www.medicalnewstoday.com/articles/312045.php, MacGill 2018). To regain the balance, different therapeutic agents are used to inhibit the gastric acid secretion or to boost the mucosal defense mechanisms by increasing mucus production, stabilizing the surface epithelial cells, or interfering with the prostaglandin synthesis (Mohod and Bodhankar 2011, Al-Radahe *et al.*, 2013, Sharath *et al.,* 2015). Plants are some of the most attractive sources of new drugs and some have shown promise for the treatment of gastro-duodenal ulcer with minimum side effects (Alexandra *et al.,* 2018). Oral administration of ASEPA was safe up to 5000 mg/kg. This attests the relative safety of the extracts with only a remote chance of acute toxicity. This result agrees with report by Ozolua *et al* (2009)

Phytochemical screening of ASEPA and fractions revealed the presence of bioactive compounds that have been previously associated with gastro protective activites. They include alkaloids, saponins, flavonoids, terpenoids, tannins, cardiac glycoside and reducing sugars. Idris *et al.* (2009) and Omodamiro *et al,* (2016) also reported the presence of these secondary methabolites in *P. americana* seed extract. Anti- bacterial and anti-fungal effects of the saponins (which were in abundance in the extract) has been documented (Lanzotti *et al.,* 2012). Saponins are believed to activate mucous membrane protective factors (Borrelli and Izzo 2000). The ability of flavonoids to increase microcirculation in the gastric mucosa has also been reported (Jarial *et al* 2018). Flavonoids act as free radical scavengers and are powerful anti-oxidants (Galleano *et al* 2010). Flavonoids and their derivatives act by decreasing lipid peroxidation by improving vascularity, slowing down the progress of cell necrosis and strengthening of collagen

fibers (Sharath, 2015). Anti bacterial effect of flavonoids has also been demonstrated (Jarial *et al.,* 2018). Tannins on the other hand, are noted for their anti-oxidant effects (Kaisarun *et al.,* 2016) and astringent property (McGee 2004). They render the outermost layer of the mucosa less permeable to chemical irritatants due to their astringent property. Tanins can also hasten the healing of wounds and inflamed mucous membrane due to their anti-inflammatory effects (Cheng *et al*., 2002) and their ability to form a protective layer over the exposed tissue hence keeping the wound from being infected (Stéphane *et al.,* 2004). Terpenoids have shown antibacterial activity and wound-healing activity (Mai *et al.,* 2003). They have also been reported to possess potent activity against gastric ulcers (Mitra *et al.,* 2014). Isolated pure form of alkaloids and their synthetic derivatives are used as medicinal agents for their analgesic, anti-inflammatory and anti-nociceptive properties (Noureddine *et al.,* 2015, Noureddine Bribi *et al.,* 2017). There was abundance of reducing sugars in this seed extract. Wang *et al.,* (2017) reported reducing sugars significantly reduced the malondialchehyde (MDA), decreased free radical activity and enhanced the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Therefore, the presence of these bioactive substances may be an indication for the antiulcer activity of the extract.

The ability of the gastric mucosa to resist injury by endogenous secretions (acid, pepsin, and bile) and ingested irritants (eg, alcohol, nonsteroidal anti-inflammatory drugs) can be attributed to a number of factors that have been collectively referred to as "mucosal defense" (Wallace 2001). The concept of gastric cytoprotection against various necrotizing agents has been routinely used to assess the antiulcer potential of different compounds. The ethanol-induced acute gastric mucosal injury model is considered to be one of the widely used experimental models of ulcer disease (Shawon and Gautam 2012). Ethanol easily penetrates the gastric mucosa and causes gastric ulcer 1 h after

administration; producing ulceration due to a decrease in gastric mucus, prostaglandin levels, glutathione, mucosal blood flow and bicarbonate secretion (Nordin, 2014). Ethanol readily penetrates the gastric mucosa due to its ability to solubilize the protective mucous and expose the mucosa to the proteolytic and hydrolytic actions of hydrochloric acid and pepsin (Oates and Hakkinen 1988) causing damage to the membrane. In the present study, ethanol-induced ulcer model was employed to confirm the gastric cytoprotective effect of the seed extract and fractions. The strong ulcer healing effect of the extract and fractions against ethanol-induced ulcer as observed by the significant ($p<0.05$) reduction in ulcer indexes might be related to the antioxidant activity of the seed which was well demonstrated in previous studies (Rodríguez-Carpena *et al.,* 2011, Owusu *et al.,* 2015, Ikpeme *et al.,* 2014). Anti-oxidants accelerate wound healing (Yen *et al.,* 2018) and compounds that act as antioxidants or activate the redox system are important for restoring gastric tissue (Hussain *et al.,* 2015).

Aspirin is known to induce ulcers by inhibiting prostaglandin synthetase in the cyclooxygenase pathway (Rainsford, 1987). Prostaglandins are found in many tissues including the stomach where they play a vital protective role via stimulating the secretion of bicarbonate and mucus, maintenance of mucosal blood flow and regulating mucosal cell turnover and repair (Hayllar and Bjarnason 1995). ASEPA and fractions conferred ulcer-protective effect against aspirin-induced ulcer as shown by the significant ($p<0.05$) reduction in ulcer indexes. The extract and fractions of *P. americana* seeds were probably exerting their activity by acting as gastro-protective agent since the underlying pathophysiology of NSAIDs-induced peptic ulcer is due to interference with mucosal prostaglandin synthesis.

Pyloric ligation-induced gastric ulceration may be attributed to stress-induced secretion of HCl in excess amounts from the parietal cells and autodigestion of mucosa by the

accumulated gastric juice (Mohod and Bodhankar 2011). Free radicals may also be associated since studies have shown changes in the antioxidant status following pylorus ligation-induced ulceration in rats (Sharath and Preethy 2015). The model is therefore used to determine anti-secretory effect of drugs that reduce secretion of gastric acid and pepsin. The antiulcer activity of ASEPA and fractions on this ulcer model was evidenced by the significant reduction in the ulcer indexes, volume of gastric acid, free acidity and total acidity of the gastric juice. The extract also increased the pH of the gastric acid. These results suggest ASEPA and fractions probably have anti-secretory property which may be responsible for the observed ulcer-healing effect.

Stress induced ulcers are produced due to the increased release of histamine, leading to an increase in acid secretion (histamine is a potent stimulator of gastric acid secretion). Increased histamine secretion also leads to increased gastrointestinal motility which can lead to folds in the GIT that may eventually lead to ulcer (Michael and Charles 1983). The ASEPA and fractions probably conferred protection against ulcers induced by cold restraint (stress) in rats by inhibition of histamine (H_1 receptor) since histamine mediate contraction in the gastric mucosa through H_1 receptors. This inhibition could result in decrease in gastrointestinal motility, leading to gastro-protection and ulcer healing.

ASEPA and fractions caused significant decrease in MDA level, confirming the cytoprtective and gastroprotective activities of the extracts. Increase in MDA levels in the stomach of the control rats suggests enhanced peroxidation leading to tissue damage and failure of the antioxidant defense mechanisms to prevent formation of excessive free radicals. Treatment with ASEPA and fractions at 500 mg/kg significantly reversed these changes. The anti-oxidant property of *P. americana* seeds has been reported in several studies (Ikpeme *et al.*, 2014, Owusu *et al.*, 2015, Francisco *et al.*, 2018 and Nurdin *et al.*, 2018). Likewise, the extract and fractions were able to preserve the catalase in the

stomach cells of the treated rats presumably by enhancing antioxidant potential of the gastric mucosa thereby preventing mucosal damage. The use of the anti-oxidant, Zinc carnosine is a novel therapeutic option in management of peptic ulcer disease (Hiraishi *et al*., 1999).

Reduction in intestinal motility ameliorates ulcer pain and hastens the healing of ulcer wounds (Al Batran *et al.,* 2013). The extract and fractions delayed the small intestinal transit in rats, an effect considered beneficial to ulcer patients. Odo *et al* (2013) also reported the anti-motility activity of *P. americana* seeds.

The photomicrographs of the stomachs of pyloric ligature and stress-induced ulcer go futher to confirm the gastoprotective and cytoprotective effects of *P. americana* seeds. The epithelium of the stomach of the negative control of pyloric ligature-induced ulcer model revealed edema and break in the epithelium. Likewise, the control for stress-induced ulcer showed discontinuity in mucosal epithelium, inflammation of the mucosa and submucosa. However treatment with ASEPA and fraction protected the stomachs of the rats in the two ulcer models. Hence, normal mucosa, submucosa and musclaris mucosa of their stomachs were observed.

Microbial colonization of the gastrointestinal system has been associated with a variety of peptic ulcer diseases and this has made antibiotics essential component in the management of the peptic ulcer disease (Amieva and Peek 2016). In this study ASEPA and fractions were found to exhibit a concentration-dependent inhibition on some microbes. The anti-microbial activity of ASEPA and fractions may have contributed to their ulcer-healing effects. Ilozue *et al* (2014) and Uzor *et al* (2016) also reported the anti-bacterial activity of *P. americana* seed extract.

The results of the in vitro study showed ASEPA and fractions exerted a non-dose dependent spasmolytic activity on the isolated guinea pig ileum. Cholinergic agents are

known to increase the secretory and motor activity of the gut (Bukhari *et al.,* 2013). The M_2 and M_3 receptors in the GIT play essential roles in the relaxation of the GIT (Ehlert *et al*., 1999). The possible mechanisms of GIT relaxation could be the inhibition of acetylcholine (Ach) by blockage of the muscarinic receptors of smooth muscles of the gastro intestinal tract. Inhibition of histamine (H_1) receptors is also a possible mechanism of the relaxant effects of these extracts since H_1 receptors are dominant in the gut and they mediate contraction (Ehlert *et al*., 1999)

As was observed especially in healing effect study, n-hexane fraction exhibited the highest ulcer inhibition and in some cases better ulcer- inhibitory effect than the standard drug cimetidine. This is in line with a report by Melese *et al.,* (2011) where the aqueous extract of *Plantago lanceolata* showed a better ulcer inhibition than ranitidine. Such findings strengthen the search for novel agents by tapping the rich herbal drugs used in folk medicine. The consistent better effect by n-hexane fraction could be as a result of the ability of the solvent to extract more of the active component from the seed which was mostly in oily form. This could also explain why polar solvents like ethyl acetate, methanol, ethanol and butanol could not fractionate the extract effectively as was observed during fractionation. This could be because the seeds contain mainly non-polar compounds as was later confirmed by the GC-MS analysis.

The HPLC identified the major compound in ASEPA and fractions as Absciscic acid. Del Refugio *et al.,* (2004) reported the isolation of two glucosylated abscisic acid derivates from avocado seeds. GC-MS analysis identified monosaturated fatty acids (alkanes) in the VLC fraction. Akpabio *et al*., (2011) had reported the presence of monosaturated fatty acids in *P. americana.* Apart from alkanes being precursor for the synthesis of many biologically active compounds, they have been reported to have anti-microbial and anti-oxidant properties (Jae-Suk Choi *et al.,* 2012, Zaha *et al*., 2016). The ability of fatty acids

to significantly inhibit gastro-intestinal motility has been documented (Zaha *et al*., 2016). The most active fraction from the VLC analysis was in oily form hence GC-MS was used to analyze the oil (volatile oil) because they were non-polar compounds. On the other hand, HPLC identifies polar compounds (Absciscic acid) which can be detected by UV light. The ulcer-healing activity of the aqeous extract and fractions of *Persea americana* seed could therefore be attributed to the combined effects of its bioactive substances.

CHAPTER FIVE

CONCLUSION, RECOMMENDATIONS AND CONTRIBUTION TO KNOWLEDGE

5.1 Conclusion

The extract and fractions of *Persea americana* seeds is relatively safe as well as having ulcer-healing and ulcer protective effects. The use of the aqueous seed extract in treatment of peptic ulcer disease in folklore medicine is justified. The ulcer healing effects could be related to the anti-bacterial, anti-secretory, cytoprotective, gastroprotective and wound-healing activities of the phytochemicals. The major compounds in the extract and fractions of *P. americana* seeds are monosaturated alkanes and Absciscic acid.

5.2 Recommendations

Many phytochemicals abound in the seed of *P. americana* that have anti-ulcer potentials. It is recommended that these bioactive compounds, especially Abscisic acid which was identified by the study as the major compound be further isolated, purified and characterized. We also recommend chronic toxicity study on this seed extract as well as further pharmacological studies on Abscisic acid with the aim of developing a novel anti-ulcer agent.

5.3 Contribution to Knowledge

The ulcer-healing and gastroprotective effects of this seed extract have been revealed through this study. This study has also exhibited the anti-secretry effect of *P. americana* seed extract as well as its invitro effects on isolated ileum tissue.

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APPENDIXES

Appendix 1.

Treatment	Dose	Ulcer index	% Protection
Control	10 ml/kg distilled water	2.75	0
ASEPA (mg/kg)	200	2.0	27
	400	1.90	31
	600	1.2	56
	800	0.75	72
N-hexane fraction mg/kg	200	1.6	42
	400	1.05	62
	600	0.9	67
Water fraction	200	1.71	38
	400	1.09	60
	600	1.03	63

Result for Determination of ED_{50} of ASEPA and Fractions

Appendix 2.

Treatment /dose	Response(mm)	Figure
NON-DOSE DEPENDENT EFFECT OF NF,WF and ASEPA		
2.5 mg NF	7	3
5 mg NF	7	3
2.5 mg WF	6	3
5 mg WF	6	3
5 mg ASEPA	14	4
10 mg ASEPA	14	4
EFFECT OF NF AND WF ON HISTAMINE CONTRACTION		
2 ug hist	66	3
2 ug hist+5 mgNF	55	3
2 ug hist+ 5 mg WF	46	3
EFFECT OF NF AND WF ON Ach CONTRACTION		
0.2 ug Ach	37	5
0.2 ug Ach+5 mgNF	32	5
0.2 ugAch+5 mg WF	32	5
EFFECT OF ASEPA ON Ach CONTRACTION		
4 ug Ach	15	4
4 ugAch+5 mg ASEPA	8	4

Effect of ASEPA and Fractions on Guinea Pig Ileum. Key: NF=n-hexane fraction, WF= water fraction, Ach= acetylcholine, Hist= histamine

Appendix 3. The Structures of the 24 Compounds from GC-MS Analysis

Methyl benzene – compound 1
Mol mass 92.06
Mol wt 92

3 methyl heptane – compound 2
Mol mass 114.14
Mol wt 114

p-dimethyl cyclohexane hexane – compound 3
Mol mass 112.13
Mol wt 112

Octane – compound 4
Mol mass 114.14
Mol wt 114

2, 6-dimethyl heptane – compound 5
Mol mass 128.16
Mol wt 128

1 ethyl 3 methyl cyclopentane – compound 6

Mol mass 112.13
Mol wt 112

1, 3-cyclopentadine – compound 7
Mol mass 106.08
Mol wt 106

Nonane –compound 8
Mol mass 128.16
Mol wt 128

Decane – compound 9
Mol mass 142.17
Mol wt 142

Decane – Compound 10
Mol mass 142.17
Mol wt 142

Decane – Compound 11
Mol mass 142.17
Mol wt 142

Tridecane – compound 12
Mol mass 184.22
Mol wt 184

Tridecane – compound 13
Mol mass 184.22
Mol wt 184

Tridecane – compound 14
Mol mass 184.22
Mol wt 184

Hexadecane – compound 15
Mol mass 226.27
Mol wt 226.45

Hexadecane – compound 16
Mol mass 226.27
Mol wt 226

2, 6, 11-trimethyl dodecane – compound 17
Mol mass 212.25
Mol wt 212

Hexadecane – compound 18
Mol mass 226.27
Mol wt 226

Hexadecane – compound 19
Mol mass 226.27
Mol wt 226

Hexadecane – compound 20
Mol mass 226.27
Mol wt 226

Hexadecane – compound 21
Mol mass 226.27
Mol wt 226.45

Hexadecane – compound 22
Mol mass 226.27
Mol wt 226.45

2-methyl nonadecane – compound 23
Mol mass 282.33
Mol wt 282

2, 4-dimethyl eicosane – compound 24
Mol mass 310.36
Mol wt 310.61