# Chapter One

# Introduction

## 1.1 Gum exudates

Gum exudates are polysaccharides colloids obtained as the natural exudates of different trees species and exhibit unique properties in a wide variety of applications in food, and nonfood industries as emulsifier, stabilizers, thickening and gelling agents, crystallization inhibitors, and encapsulating agents, in pharmaceutical, cosmetic, ink, paper, textile and other industries (Nussinovitch, 1997; Tan, 2004; Whistler, 1993). Nowadays, the most important types are those produced by *Acacia senegal* and *Acacia seyal*, *Anogeissus latifolia* (gum ghatti or Indian gum), *Astragalus gummifer* species (gum tragacanth) and by *Sterculia urens* (gum karaya) (Kumbhare & Bhargava, **1999**; Lapasin & Pricl, **1995**). Gum Arabic a natural hydrocolloids is a water-soluble polysaccharide, from vegetable source is defined as the gummy exudates obtained from the trees of *Acacia Senegal* var. *Senegal*. It is the oldest and the best known of all natural gums. *Acacia Senegal* gum shows, however, good functional properties that clearly differ from those of other hydrocolloids such as Guar Gum or Agar-Agar (Tan, 2004). *Acacia Senegal* gum is the most important gum exudates considering its unique properties and commercial value. It represented roughly 4% of the hydrocolloid market in 2000, while the markets of gums karaya and tragacanth were marginal. (Williams and Phillips, 2000). Gum Arabic was notoriously unstable in supply and price, for this reason, and also as a consequence of the need to search for new renewable raw materials capable of yielding natural hydrocolloids with added value, there is endless efforts and interest in testing and the properties of new gum exudates; (Williams and Phillips, 2006).

Furthermore, since the major uses of gums are in food, pharmaceutical, medicinal and cosmetic, targeting gums safety and purity issues are very important aspects of research.

## 1.2 Aim of study

This study focuses on the physicochemical characterization and toxicological evaluation of gums obtained from *Combretum glutinosum* tree of Sudanese origin (habil gum). Little is known about the chemistry of this gum and no toxicological study has been undertaken. The present study aims at realizing the following objectives:

1. To characterize the gum from *Combretum glutinosum* using standard physico-chemical methods.
2. To under- take toxicological study of *Combretum glutinosum* gum using cytotoxicity techniques.
3. To establish a standard specification for *Combretum glutinosum*.

# Chapter Two

## Literature Review

### 2.1 *Combretum glutinosum*

*Combretum glutinosum* gum is a natural colloids from vegetable source, it is defined as the gummy exudates which obtained by incision the bark of trees of *Combretum glutinosum,* which is the type genus of family *Combretaceae* . It is small savanna tree, durable for about 2years (Sahni, 1968). Its is fast growing species ,widely distributed and often abundant and gregarious .It is particularly resistant to arid condition surviving where grasses will not, and it recovers quickly from burning. (Jansen and Marquet 2005).

#### 2.1.1 The Family *Combretaceae*

The family *Combretaceae* belongs to the order Myrtales and consists of 600 species of trees and shrubs in 20 genera, which include 3 sub-tribes *Thiloa Eichl*. (3 spp.), *Preleopsis* (approx. 10 spp.) and *Termindia* L. (approx. 150 spp.) and are found through the tropics and sub-tropics. The largest genus is *Combretum*, with about 370 species, while *Terminalia,* the second largest, has about 150 species. They occur in most parts of Africa and are often the dominant vegetation. The other genera are much smaller, including as example *Calopyxis* and *Buchenaoia* which have 22 species for each one and *Quisquulis, Anogeissus*, have 16, 14, species, respectively (Angeh, 2006).

There were several attempts at taxonomic classification in the nineteenth century. Engler and DieIs (Engler and Diels, 1899) divided the species on a world-wide basis into 55 sections in 1899; their classification still forms the basis for modern revisions, such as Exell's account of the American species. A revision by Exell and State in 1966 grouped EngIer and Diel's sections, and 10 sections added by other authors, into subgenera, the major of which is subgenus *Combretum*, subgenus *Cacoucia*, and subgenus *Apetalanthum* (Figure 2.1.). In 1969, Exell and Stace described 3 new sections (all African species) of the sub-genus *Combrerum*;

this brought the total number of sections in the genus to 68, of which 22 in subgenus *Combretum* and 5 in subgenus *Cacoucia* are recognised in Africa ( Exell and Stace, 1966; Exell and Stace, 1969 ). These taxonomic difficulties have arisen because the genus *Combrerum* is a complex, heterogeneous population, in which there appears to be continuous reshuffling of genes (Exell, 1970).

### 2.1.2 Botanical nomenclature of *Combretum*

The genus *Combretum* Loefl. is the largest in the Family *Combretaceae* (order, Myrtales); it is cosmopolitan in the tropics and sub-tropics, although absent (Stace, 1969) from Australia and the Pacific Islands. The naming of species in *Combretum* has long been recognized (Exell, 1953) as a difficult problem, especially in the absence of flowers. Over 600, specific, names are now known to have been used to represent some 250, actual, species; consequently, the synonymy is often, unusually, extensive .The greatest range of structure and, most of the difficult, taxonomic problems are found in Africa, where about180, of the known species, occur; there are around 30 Asian species (Stace, 1973).

**Figure 2.1 TAXONOMIC CLASIFICATION OF THE FAMILY *COMBRETACEAE***

**FAMILY** → **SUB-FAMILIES**

**COMBRETACEAE**

**(ORDER, MYRTALES)**

**(a) STREPHONEMATOIDEAE Engl. and Diels**

**[ l genus: Srrephonema Hook. f. (7 spp.)]**

**(b) COMBRETOIDEAE (10 genera)**

**Tribe**

**COMBRETEAE (16 genera)**

**Tribe**

**LAGUNCULARIEAE Engl. and Diels**

**3 genera: Lagrmcdnria Gaertn. f. (l-2 spp.)**

**Lumnirzeru Willd. (2 spp.)**

**Macropteranthes F. Muell (4 spp.)**

**SUB-TRIBE**

**[Thiloa Eichl. (3 spp.)**

**Quisquulis L. (16 spp.)**

**calopyxis Tul. (22 spp.)**

**Meiostemon Exe11 and State (2 spp.)**

**Guieru Juss. (1 spp.)**

**Culycopteris Lam. (1 spp.)**

**Combrefum Loefl. (approx. 200 spp.)]**

**SUB-TRIBE**

**[Preleopsis**

**Engl. (10 spp.)]**

**SUB-TRIBE**

**[Termindia L. (approx. 150 spp.)**

**Ramatuella Runth (6 spp.)**

**Terminaliopsis Danguy (1 spp.)**

**Bucida L., (9 spp.)**

**Buchenaoia Eichl. (22 spp.)**

**Anogeissus Wall. (14 spp.)**

**Finetia Gagnop. (1 spp.)**

**Conocarpus L., (2 spp.)]**

**SUB-GENUS**

**Combretum**

**SUB-GENUS**

**Cacoucia**

**SUB-GENUS**

**Apetalanthum**

**(Further sub-divided into a total of 68 sections)**

### 2.1.3 Distribution

*Combretum glutinosum* is distributed through out the whole sahel belt of Africa, in many parts of eastern and southern tropical Africa and in India (El Amin 1990). It is also wide spread all over West Africa from Senegal to Cameroon and extends to Sudan (Vogt, 1989). In Sudan the number cited are for *C. cordofanum* which is the synonym of *C.glutinosum* and found in Kordofan state, Nubba mountains Jebel Mara. (Sahni, 1968)

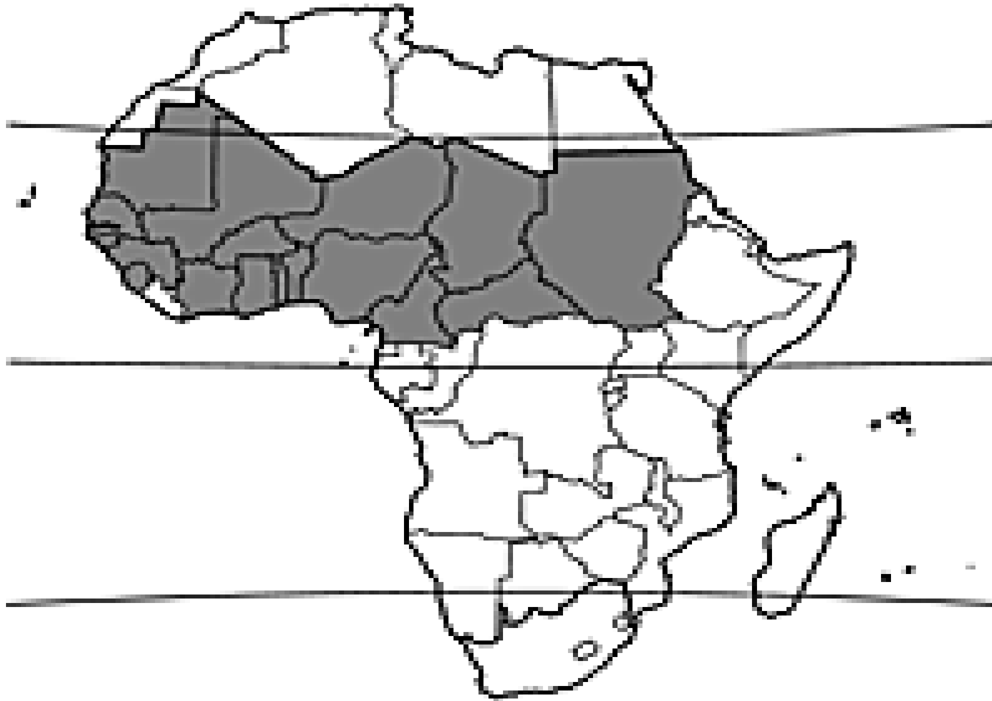


**Figure.2.2 Map of Africa showing *Combretum glutinosum* trees Belts**

### 2.1.4 Botanical of Classification of *Combretum glutinosum*

Kingdom: *plantae*

Order: *Myrtales*

Family: *Combretaceae*

Latin name: *Combretum glutinosum* Perr.ex DC

Common synonym(S): *Combretum cordofanum* Engl. and Diels

Arabic: Habil, Habil elmur,Um hebila

### 2.1.5 Description

Small trees 7-10 m high. Bark grey, smooth and some times scaling into dark plates. Leaves opposite or sub opposite, elliptic, thich, coriaceous, glutinous about 17.5×7.5 cm, petioles hairy 1 cm long ,inflorescence axillary panciles 4-5 cm long , flowers 4-merous greenish brown, densely hairy, pedicels hairy,1.5 mm long. Fruit: ovate angular, 4-winged, 1-seeded, 2.5-3.5 ×2 cm, truncate at base, puberulous. Flowers: December – March, fruits April. (El Amin 1990).

### 2.1.6 Tree requirements

Rainfall: Low annual rainfall about 300-700 mm.

Soil type: Prefers sandy and free-draining soils.

Propagation: About 20,000 seeds/kg. (Vogt, 1989).

### 2.1.7 Growth and development

In the savanna zone *Combretum glutinosum* is evergreen, in the Sahel its leaves fall for a few months during the dry season. Flowering is in the dry season after bush fires to which it is very resistant. The timing of flowering and fruiting varies with location. In Burkina Faso and Mali flowers develop from December to March (mid to late dry season), but can continue until July. Fruiting begins in January, and continue until November, although it is often much shorter- from March until May (late dry season). (Steentoft, 1988). In Sudan flowers appears in December-March, fruits in October-December (Sahni, 1968)

A

B

**Figure.2.3** ***Combretum glutinosum*** **(A) Fruiting tree, and (B) Flowering branch.**

### 2.1.8 Production and international trade

In Mali the increased interest and demand has made bogolan production (which is a black colour being due in all cases to the reaction of the various tannins present in the plant with iron compounds in the mud) an important economic activity with centers spreading from Mali to neighbouring countries, and with international markets, mostly in Europe and the United States .Wood ash from *Combretum glutinosum,* widely used in indigo dyeing, is traded for this use both at regional and international scale but statistics are not available . (Jansen and Marquet, 2005).

### 2.1.9 Chemical constituents

An analytical study of some specimens of *combertum* and from them *Combretum glutinosum* showed that the botanical classification and synonymy, and the chemistry, of this genus are more complex. Gum present in the bark; consist of uronic acid compounds which hydrolyze to a number of sugars. It consist of Glucuronic acid, galacturonic acid, 4-*0*-methylglucuronic acid, galactose, arabinose, and rhamnose .( Anderson , *et al*1977)

### 2.1.10 Uses

It is used mainly for firewood and charcoal, the yellowish wood is hard and extremely durable and is suitable for fencepost, framework for huts, tool handles and other general carpentry. Extracts from the bark, leaves and roots give a yellow dye. Many medicinal uses have been reported: the bark for treating influenza and rheumatism, and for dressing wounds; the leaves as a remedy for malaria, headaches, rheumatism, colic and sores. Other parts of the tree have been used in deriving a medication for syphilis and boils. (Vogt, 1989). Combreglutinin is one of tannins that have been isolated from the leaves of *Combretum glutinosum* which has interesting medicinal properties particularly for the treatment of hepatitis B. Other proven medicinal effects are promoting discharge of urine, reduction of hypertension, discharge of bladder stone and antitussive and disinfectant properties of leaves. (Jansen and Marquet, 2005).

In West Africa from Senegal to Cote d' Ivoire the leaves, stems and root bark of *Combretum glutinosum*, collected from the wild, are important sources of yellow to brownish yellow dyes for cotton textiles. On the other hand in Senegal the gum of the bark is used to fill the cavity of tooth.In Burkina Faso, Benin and Nigeria, these dyes are also used to dye leather and mats made of various vegetable fibers. In Mali various tannins present in the plants made reaction with the iron compounds of the mud to produce black dyes for cotton textiles.In traditional medicine *Combretum glutinosum* is highly valued. A decoction or infusion of the leaves, bark or fruits is very popular, mainly to treat urinary, liver and kidney complaints, also all kinds of respiratory problems, fevers, intestinal complaints and to clean wounds and sores . (Jansen and Marquet 2005).

**2.2 Toxicity:** is defined as a degree to which a substance is poisonous or can cause harm to exposed humans or animals. In other words, can be defined as sum of adverse effects or degree of danger posed by a substance to living organisms. Toxicity can refer to the effect on whole organism, such as humans, animals, bacterium, or plant, as well as the effect on a substructure of the organism, such as a cell (Cytotoxicity) or an organ (Organotoxicity). (Glibert 2001).

### 2.2.1Classification of toxicity

Frequency of exposure refers to the number of times a person is exposed to a toxic material and duration to the time between exposures. Duration of exposure can be acute, sub chronic, or chronic.

Acute exposure may be defined as a single exposure to the substance which may result in severe biological harm or death; a cute exposure is usually characterized as lasting no longer than a day.

Chronic exposure means harmful effects produced through repeated or continuous exposure over an extended period.

Sub chronic exposure is defined as harmful effects produced through repeated or continuous exposure over twelve month or more but less than the normal lifespan of organism. (Glibert, 2001).

**2.2.2 Safety and toxicity of gums**

Acacia gum, is traditionally utilized by African and Indian populations to prevent and treat intestinal disorders. There is evidence that gum feeding can improve intestinal transit and provide digestive comfort. Such traditional health promoting characteristics of dietary fiber have been used and accepted. It is given to lactating mothers in India as part of a special diet and in medical practice used in the treatment of diarrhea and diabetes. Interestingly, apes and monkeys also frequently eat plant gum exudates (Phillips *et al* 2008).

Gum Arabic is approved for use as food additives by the US Food and Drug Administration and is on the list of substances that is a generally recognized as safe with specific limitations. The potential toxicity of gum acacia has been extensively studied. The oral $LD_{50}$ values for mice, rats, hamsters, and rabbits are 16, 18, 16, and 8 g/kg/day, respectively (JECFA, 1982). Genotoxicity studies indicate that gum acacia is not genotoxic (JECFA, 1982; Sheu *et al*., 1986). Extensive investigations concerning the safety of conventional preparations have shown no subchronic toxicity (Anderson *et al*., 1982; JECFA, 1982), chronic toxicity or carcinogenicity (Melnick et al., 1983). No compound-related effects were noted in teratology studies in rats, mice, hamsters, and rabbits (Collins *et al*. 1987; JECFA, 1982). Long-term toxicity/carcinogenicity studies of gum acacia in rats (Anderson *et al*., 1982; Doi *et al*., 2006; Melnick *et al.*, 1983) and mice failed to demonstrate any significant compound- related effects. On the other hand no toxicological studies what so ever have been undertaken for *Combretum glutinosum* gum, so the present study under take toxicology of *Combretum glutinosum* gum by cytotoxicity technique.

**2.2.3 Cytotoxicity** usually refers to the degree to which something is toxic to living cells, denoting certain drugs used in the treatment of cancers. The prefix cyto- denotes a cell. It comes from the Greek kytos meaning hollow, as a cell or container. Toxic is from the Greek toxikon meaning arrow poison.

**2.2.3.1 Principles and Cell physiology**

Treating cells with a cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lyses. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death (apoptosis). ( *Promega Corporation 2006*)

Apoptosis is characterized by well defined cytological and molecular events including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation and cleavage of DNA into regularly sized fragments. *( Promega Corporation 2007*),Therefore Assays are available to measure a variety of different markers that indicate the number of dead cells (cytotoxicity assay), the number of live cells (viability assay), the total number of cells or the mechanism of cell death (e.g., apoptosis).Assay responsiveness to test compounds can be influenced by many subtle factors including culture medium surface-to-volume ratio, gas exchange, evaporation of liquids and edge effects. These factors are especially important considerations when attempting to scale up assay throughput. A basic understanding of the changes that occur during different mechanisms of cell death helps in which endpoint to choose for a cytotoxicity assay (Riss and Moravec, 2004).

**2.2.3.2 Evaluation of cytotoxicity**

Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. Researchers can either look for cytotoxic compounds, assessing cell membrane integrity is one of the most common ways to evaluate cell viability and cytotoxic effects, for example

**1-Vital dyes, such as trypan blue or propidium iodide:** Vital dyes used for compounds that have cytotoxic effects often compromise cell membrane integrity. Membrane integrity can be assessed by monitoring the passage of substances that are normally sequestered inside cells to the outside. (Riss and Moravec, 2004)

**2-lactate dehydrogenase (LDH):** LDH is one commonly measured molecule which measure relative numbers of live and dead cells within the same cell population. (Decker T and Lohmann, 1988). The live-cell protease is only active in cells that have a healthy cell membrane, and loses activity once the cell is compromised and the protease is exposed to the external environment. The dead-cell protease cannot cross the cell membrane, and can only be measured in culture media after cells have lost their membrane integrity. (Niles AL, Moravec RA. *et al.* 2007)

3-ATP assay: ATP-based assays include bioluminescent assays in which ATP is the limiting reagent for the luciferase reaction (use ATP content as a marker of viability. (Fan and Wood 2007).

4-MTT or MTS assay: These are colorimetric assays fore measuring the activity of enzymes that reduce MTT or close dyes (XTT,MTS,WSTs) to formazan dyes, giving a purple color (Riss and Moravec, 2004).

### 2.2.3.3 The half maximal inhibitory concentration ($IC_{50}$)

The half maximal inhibitory concentration ($IC_{50}$) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half. In other words, it is the half maximal (50%) inhibitory concentration (IC) of a substance (50% IC, or $IC_{50}$). It is commonly used as a measure of antagonist drug potency in pharmacological research. Sometimes, it is also converted to the $IC_{50}$ scale (-log $IC_{50}$), in which higher values indicate exponentially greater potency. According to the FDA, $IC_{50}$ represents the concentration of a drug that is required for 50% inhibition *in vitro*.

It is comparable to an $EC_{50}$ for agonist drugs. $EC_{50}$ also represents the plasma concentration required for obtaining 50% of a maximum effect *in vivo*. (Swanson *et al*1990)

**2.2.3.4Determination $IC_{50}$ of a substance**

The $IC_{50}$ of a drug can be determined by constructing a dose-response curve and examining the effect of different concentrations of antagonist on reversing agonist activity. $IC_{50}$ values can be calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist. $IC_{50}$ values are very dependent on conditions under which they are measured. In general, the higher the concentration of inhibitor, the more agonist activity will be lowered (Swanson *et al*1990). Assignment to cytotoxicity class is based typically on results of $IC_{50}$ values as shown in Table (2.1)

**Table 2.1: Classification of the cytotoxicity for natural ingredients (Balantyne, 1999). Cited in (Shiraz *et al.* 2004).**

| **Category** | **$IC_{50}$** |
|---|---|
| Potentially very toxic | $IC_{50}$<10μg $mL^{-1}$(million cells $mL^{-1}$) |
| Potentially toxic | 10μg $mL^{-1}$< $IC_{50}$<100μg $mL^{-1}$ |
| Potentially harmful | 100μg $mL^{-1}$< $IC_{50}$<1000μg $mL^{-1}$ |
| Potentially non toxic | $IC_{50}$>1000μg $mL^{-1}$ |

**2.2.3.5 *In vitro*** (Latin: *within glass*) refers to studies in experimental biology that are conducted using components of an organism that have been isolated from their usual biological context in order to permit a more detailed or more convenient analysis than can be done with whole organisms. In contrast, the term *in vivo* refers to work that is conducted with living organisms in their normal, intact state. Common examples of *in vitro* experiments include (a) cells derived from multicellular organisms (cell culture or tissue culture), (b) subcellular components

(e.g. mitochondria or ribosomes), (c) Cellular or subcellular extracts (e.g. wheat germ or reticulocyte extracts), or (d) purified molecules in the test tube (often proteins, DNA, or RNA) either individually or in combination (Alberts, 2008).

**2.2.3.5.1 Advantages of *in vitro* studies**

Living organisms are extremely complex functional systems that are made up of, at a minimum, many tens of thousands of genes, protein molecules, RNA molecules, small organic compounds, inorganic ions and complexes in an environment that is spatially organized by membranes and, in the case of multicellular organisms, organ systems. For a biological organism to survive, these myriad components must interact with each other and with their environment in a way that processes food, removes waste, moves components to the correct location, and is responsive to signaling molecules, other organisms, light, sound, temperature and many other factors. This extraordinary complexity of living organisms is a great barrier to the identification of individual components and the exploration of their basic biological functions. The primary advantage of *in vitro* work is that it permits an enormous level of simplification of the system under study, so that the investigator can focus on a small number of components (Naim et al. 2009). For example, the identity of proteins of the immune system (e.g. antibodies), and the mechanism by which they recognize and bind to foreign antigens would remain very obscure if not for the extensive use of *in vitro* work to isolate the proteins, identify the cells and genes that produce them, study the physical properties of their interaction with antigens, and identify how those interactions lead to cellular signals that activate other components of the immune system (Geoffrey, 2009).

**2.2.3.5.2 Disadvantages of *in vitro* studies**

The primary disadvantage of *in vitro* experimental studies is that it can sometimes be very challenging to extrapolate from the results of *in vitro* work back to the biology of the intact organism. Investigators doing *in vitro* work must be careful to avoid over-interpretation of their results, which sometimes lead to erroneous

conclusions about organismal and systems biology (Rothman, 2002). For example, scientists developing a new viral drug to treat an infection with a pathogenic virus (e.g. HIV-1) may find that a candidate drug functions to prevent viral replication in an *in vitro* setting (typically cell culture). However, before this drug is used in the clinic, it must progress through a series of *in vivo* trials to determine its safety and effectiveness in intact organisms (typically small animals, primates and humans in succession). Many candidate drugs that are effective *in vitro* prove to be ineffective *in vivo* because of issues associated with drug delivery to affected tissues, or toxicity towards essential parts of the organism that were not represented in the initial *in vitro* studies (De Clercq, 2005).

**2.2.3.6 *In vivo*** (Latin for "within the living") is experimentation using a whole, living organism as opposed to a partial or dead organism, or an *in vitro* ("within the glass", i.e., in a test tube or petri dish) controlled environment. Animal testing and clinical trials are two forms of *in vivo* research. *In vivo* testing is often employed over *in vitro* because it is better suited for observing the overall effects of an experiment on a living subject. This is often described by the maxim *in vivo* veritas. In microbiology *in vivo* is often used to refer to experimentation done in live isolated cells rather than in a whole organism, for example, cultured cells derived from biopsies. In this situation, the more specific term is *in vivo*. Once cells are disrupted and individual parts are tested or analyzed, this is known as *in vitro* (Jeffrey 2007).

**2.2.3.7 Correlation between basal cytotoxicity and Acute Lethality**

Acute systemic toxicity studies have been widely conducted on rodents to determine the relative health hazard of chemicals and products. Acute oral toxicity testing is typically the first step in identifying and characterizing hazards associated with a particular chemical. Historically, lethality estimated by the median lethal dose ($LD_{50}$) test has been a primary toxicological endpoint in acute toxicity tests.( Garle *et al.*,1994; Halle, 2003)

Recent studies have shown a Correlation between *in vitro* and *in vivo* acute toxicity. These studies suggest that *in vitro* methods may be helpful in predicting in vivo acute toxicity. An International Workshop on *In vitro* methods for assessing acute systemic toxicity was convened on 2000 ( Guidance 2001) to review the validation status of available *in vitro* methods for predicting acute toxicity, and to develop recommendations for future research and development efforts that might further enhance the use of *in vitro* for assessments of acute systemic toxicity. The workshop was organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP), of the Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), The U.S. Environmental protection Agency (U.S.EPA), the NTP, and the National Institute of Environmental Health Sciences (NIEHS) sponsored the workshop. Breakout Groups, comprised invited scientific experts and ICCVAM agency scientists, their recommendation charged the current validation status of basal cytotoxicity methods. Their conclusions were based on several studies but, primarily, those by Drs. Horst Spielmann and Willi Halle, and their colleagues at the German Centre for the Documentation and Evaluation of Alternatives to Testing in Animals. Halle compiled a Registry of cytotoxicity (RC) containing *in vivo* acute toxicity data and *in vitro* cytotoxicity data for 347 chemicals. The data were used to construct a regression model to estimate $LD_{50}$ values from cytotoxicity data. They subsequently proposed that using these estimates as starting doses for *in vivo* acute toxicity studies such as the Up-and- Down procedure or the Acute Toxic Class method could reduce the number of animals used by as much as 30 percent.

The RC has made a major contribution to the knowledge of correlation between *in vitro* cytotoxicity and *in vivo* lethality (Halle and Spielmann,1992; Halle 1998).

The RC data clearly demonstrate a strong relationship between *in vitro* cytotoxicity and acute lethality in rodents, according to the following linear regression model,

**$\text{Log}(LD_{50}) = 0.435 \times \text{Log}(IC_{50}) + 0.625$; $r = 0.67$ (Halle 1998).**

#### 2.2.3.7.1 Procedure for qualifying a cytotoxicity test for use with the registry of cytotoxicity (RC) prediction model

Before using a candidate *in vitro* cytotoxicity test to predict starting doses, the correlation between *in vitro* and *in vivo* tests must be established quantitatively. This can be achieved either by:

1- *In vitro* testing of a large number of chemicals with known $LD_{50}$ values and deriving a regression formula based on the correlation between the *in vitro* and *in vivo* data.

2- Testing a smaller number of chemicals and applying Halle's RC prediction model (regression formula ), which is derived from the correlation of *in vitro* and *in vivo* data . (Halle 1998; spielmann et al., 1999).

In the later case, the performance characteristics of the new method should be determined and compared with those of the RC information, by suggesting a set of reference chemicals that should be tested with the candidate *in vitro* cytotoxicity method. The resultant regression line should then be compared with that of the current RC regression line. If the line falls with ± log 5 boundaries of RC regression line, hence regression parameters of RC may be used to predict the $LD_{50}$ starting doses.

ICCVAM list 20 different test protocols for basal cytotoxicity, the most promising candidate cytotoxicity test is BALB/c 3T3 fibroblasts cell NRU test and MITT and Neutral Red Uptake cytotoxicity test (NRU). The later using both mouse fibroblast cell line( BALB/c 3T3) and primary normal human keratinocytes (NHK) for predicting the $LD_{50}$ starting doses for Acute oral systemic toxicity tests (ICCVAM 2001).

**2.2.3.7.2 Predicting $LD_{50}$ starting doses:**

The $IC_{50}$ (in mmol/l) for the trial chemical is entered into the regression equation to calculate an $LD_{50}$ in mmol/Kg. Multiplying by the molecular weight of the trial chemical transforms the mmol/Kg value into mg/kg.

The new regression lines obtained with NHK and 3T3 cells are within the $\pm$ log 5 interval of the RC, and, though slightly steeper, are almost parallel to RC regression function, thus intercepts and regression coefficients of the experimentally obtained new regressions do not differ significantly from the literature-based RC regression equation:

**RC regression: Log (LD50) = 0.435× Log (IC50) + 0.625 ;( r = 0.67)**

**New NHK NRU regression: Log (LD50) = 0.498× Log (IC50) + 0.551; (r = 0.9356)**

**New 3T3 NRU regression: Log (LD50) = 0.506× Log (IC50) + 0.475; (r = 0.9848)**

# Chapter Three

# Materials and Methods

## 3. Materials

Twenty samples of *Combretum glutinosum* gum samples were collected from Blue Nile, (Eldamazaein area). The samples were large whole nodules and tears with colours ranging from dark brown, to pale brown. The nodules break with glassy fracture. Samples were collected during two consecutive seasons 2007, 2008.

**Figure.3.1 Nodules gum of *Combretum glutinosum***

## 3.1 Sample preparation for analysis:

The samples of the gum were prepared according to AOAC (2000). Any foreign particles were removed from the samples before starting analysis. The gum samples were ground using a mortar and pestle, into fine powder that passed-through thieve No. 0.5, and, subsequently, kept in sterile, screw capped, polyethylene containers.

### 3.2 Physicochemical characterization methods

#### 3.2.1 Moisture content %

The Moisture content was measured using moisture analyzer instrument (AND MX-50, Japan, A&D Company limited).The operations of instrument was based on the principle of thermogravimetrc analysis. The analyzer was built using sensitive super hydride sensor (S.H.S) adopted in an analytical balance. A 400W halogen lamp was used as the heating source and the temperature on the sample pan can reach 200 °C within two minutes. Therefore, the results were precise and reproducible. The analyzer results give % moisture content in the sample by the difference between the wet weight and dry weight.

**Procedure**

Two grams of the ground *Combretum glutinosum* sample were weighted in moisture analyzer, then it was spread on the pan, the instrument was set at 105°C the analysis was started. Then the moisture percentage was read directly from the instrument display. The procedure was repeated three times and the average of the three determinations was calculated for each sample.

#### 3.2.2 Total ash %

Two grams of sample were accurately weighed in a dried, pre- weighed crucible. The crucible and its contents were ignited in muffle furnace (Nabertherm, Germany) set at 550°C for five hours. It was then cooled in a desiccator and re-weighed. Total ash was calculated as a percentage from the original weight of *Combretum glutinosum* gum sample on dry weight basis. (FAO. 1991).

#### 3.2.3 pH value:

pH was determined for 1% aqueous solution using WPA CD 510 pH meter at room temperature.

#### 3.2.4 Detection of Starch and dextrin

A gum solution (2%) was boiled, allowed to cool then few drops of iodine

solution was added. No bluish or reddish colour was observed, indicating absence of starch (FAO. 1991).

### 3.2.5 Detection of Tannin

To 10 ml of 2% solution of the gum sample about 0.1 ml of $FeCl_3$ (6% w/v) solution was added. Appearance of a blackish coloration or blackish Precipitate indicates presence of tannins. No such Precipitate was observed (FAO. 1991).

### 3.2.6 Solubility measurement of *Combretum glutinosum* gum

1g *Combretum glutinosum* gum was stirred in 100 $cm^3$ water or electrolyte solution overnight. The dispersion was centrifuged at 3000 rpm for 45min and the supernatant layer was drawn off with a dropper. 30 ml of Distilled water was added to the insoluble fraction, which was stirred and recentrifuged at 3000 rpm for 10min and the clear supernatant added to the soluble fraction. This water wash was repeated several times to remove electrolyte salts, and then the insoluble fraction was dried at 105$^{o}$C and weighed. The solubility of *Combretum glutinosum* gum was tested in several solvent, Water, EDTA and $Na_2CO_3$ (Jefferies *et al*., 1978; Jefferies *et al*., 1981).

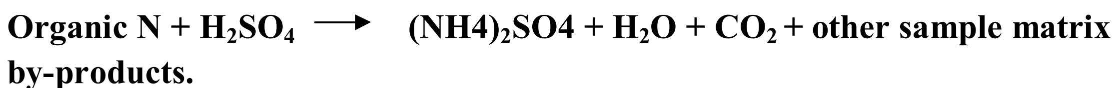
### 3.2.7 Nitrogen and crude protein%

The Kjeldahl method was developed over 100 years ago for determining the nitrogen contents in organic and inorganic substances. Although the technique and apparatus have been modified over the years, the basic principle introduced by Johan Kjeldahl remains the same (AOAC, 1995). The method was performed under three main steps: digestion, distillation, and titration.

#### 3.2.7.1Digestion

The method involves block digestion of the sample in sulphuric acid to convert the protein nitrogen to ammonium sulphate. The boiling point is elevated by the addition of potassium sulphate.The elevated boiling temperature is necessary to break the peptide bonds and convert the amino groups in protein to ammonium ions. Copper catalyst is added to enhance the reaction rate.

The general equation for the digestion of an organic sample is shown below:

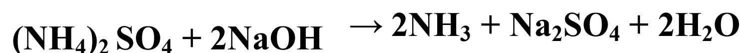


**Organic N + $H_2SO_4$ $\longrightarrow$ $(NH4)_2SO4$ + $H_2O$ + $CO_2$ + other sample matrix by-products.**

### 3.2.7.2 Distillation

After the digestion, the digest-mix is diluted with water to avoid mixing concentrated acid and to prevent the digest from solidifying.

Ammonia is then liberated by alkaline distillation to convert $NH_4$ to $NH_3$ as indicated in the following equation.



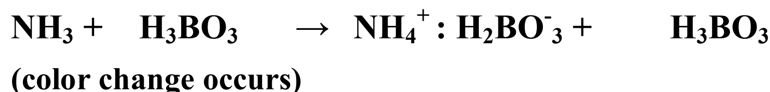
$$(NH_4)_2\,SO_4 + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

### 3.2.7.3 Titration

Titration quantifies the amount of ammonia in the receiving solution. The amount of nitrogen in a sample can be calculated from the quantified amount of ammonium ion in the receiving solution.

There are two types of titration—back titration and direct titration. Both methods indicate the ammonia present in the distillate with a color change.

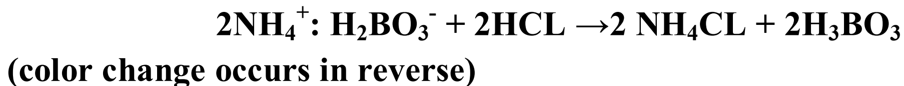
In direct titration, if boric acid is used as the receiving solution instead of a standardized mineral acid, the chemical reaction is:



$$NH_3 + H_3BO_3 \rightarrow NH_4^+ : H_2BO^-_3 + H_3BO_3$$

**(color change occurs)**

The boric acid captures the ammonia gas, forming an ammonium-borate complex. As the ammonia collects, the color of the receiving solutions changes.



$$2NH_4^+ : H_2BO_3^- + 2HCL \rightarrow 2\,NH_4CL + 2H_3BO_3$$

**(color change occurs in reverse)**

Then titrated with a stronger standardized hydrochloric acid, using colorimetric end-point detection.

#### 3.2.7.4 Procedure

The digestion block was Turn on and heat to 420 °C, triplicate 1 gram of *Combretum glutinosum* were, accurately, weighed in kjeldahl flasks, and Kjeldahl tablets were added to each flask, then twelve ml of, nitrogen free, concentrated sulphuric acid were added to each flask, and they were mounted on a kjeldahl digestion system capped with aerated mainfold and heated at 420 °C until a clear pale yellowish – green solution was obtained, indicating complition of digestion. The digestion time is approximately 60 minutes. The flasks were allowed to cool to room temperature, and their contents were transfered, quantitatively, to a kjeldahl distillation apparatus. Kjeltec system (Kjeltec 2400, Foss, Sweden) which was used in this study had automatic steam distillion and automatic titration units. The system were added 80 ml of distilled water, then 50ml of 40% sodium hydroxide were, carefully, introduced into the distillation apparatus and steam distillation of ammonia was commenced. Released ammonia was absorbed in 30 ml of 1% boric acid. Borate ions generated were back titrated against 0.01M hydrochloric acid using screened methyl red indicator.

A set of blanks was curried out simultaneously in the same way.

The percentage of nitrogen content of the sample was calculated as follows:

$$N\ \% = \frac{14.01 \times M\ (V_1 - V_2) \times 100}{W \times 1000}$$

Where

M = molarity of hydrochloric acid, $V_1$ = volume of titrant for sample.

$V_2$ = volume of titrant for blank and W = weight of sample.

The percentage of protein (%P) was calculated from nitrogen content using Nitrogen Conversion Factor (NCF), 6.6 as follows:

$$\%P = \%N \times 6.6$$

### 3.2.8 Determination of Metals

Determinations of metals was made with Atomic Absorption spectrophotometer (GBC/ Avanta/ Austuralia) equipped with deuterium arc and background correction. The elements of interest were to be determined using, conventional hollow cathode lamps. The operating parameters are those recommended by the instrument manufacturer, where burner position, flame conditions and aspiration rate were optimized for maximum absorption.

#### 3.2.8.1 Procedure

##### 3.2.8.1.1 Optical parameters and preparation of Standard Curves

Series of standard solutions of trace metals were prepared by suitable dilutions of stock solution in concentration ranges that were expected in the sample solution. The instrument setting (wave length of spectral line, hallow-cathode lamp current, slit width and flame type) were optimized for each element to be measured. A calibration curve for each element was obtained by plotting the absorbance against the concentration, the curve was used to determine the concentration of specific element in the sample solution.

In order to bring the samples into solution, wet digestion method using microwave digestion was carried out (Ethos advance microwave digestion system – Milestone).

##### 3.2.8.1.2 Wet digestion by microwave method

A 0.2g of *Combretum glutinosum* were weighted accurately in a vessel and then covered with its lid. A 6.0 ml of conc. $HNO_3$ were added and the mixture was allowed to stand at room temperature until initial reaction subsided and then 2.0 ml of hydrogen peroxide were also added. The solution was then transferred to the microwave unit after closing the vessel inside the rotator, and then the microwave program was started by setting the time and watt. The vessels was cooled and opened, then rinsed with deionized water three times in a beaker as well as beaker side. This solution was filtered in 25.0 ml volumetric flask and completed to the mark with deionized water, and transferred to a polyethylene container.

#### 3.2.8.1.3 Atomic absorption spectrometer- Measurement of samples for metals

A blank solution for samples was also prepared in the same way as the sample solution. The net absorbance of analyte in the sample solution was obtained by subtracting the absorbance of blank solution from that of the analyte in the sample solution. The metal ion concentrations of samples were read on (AAS-GBC Avanta Australia), (Zhou. *et al* 1996).

### 3.2.9 Determination of acid equivalent weight and uronic acid:

Uronic acid is widely distributed in animal and plant tissues. They constitute a major component of many natural polysaccharides. Different methods have been developed for determination of uronic acid.

These include colorimetric,decarboxylation and acid-base titrimetric Methods(Abdelrahman, 2008).

#### 3.2.9.1Procedure

Acid equivalent weight was determined according to the method reported in Encyclopedia of Chemical Technology (1966) with some modification. A cation exchange column packed with amberlite-120 resin $H^+$ (acid form) was thoroughly washed with 2 M sulphuric acid, left to stand for 10 minutes, then continuously, washed with 150cm$^3$ distilled water until free of sulphate . The presence of sulphate was checked by testing with barium chloride (saturated solution). 50 cm$^3$ of 1% gum solution were introduced on the top of the column and allowed to elute under gravity action . The elution rate was adjusted to one drop per 2second. The eluent and washing were collected and titrated against standard 0.1 M solution of sodium hydroxide using phenolphthalein indicator.

$$Acid\ equivalent\ weight = \frac{weight\ of\ the\ sample \times 1000}{volume\ of\ the\ titrant \times molarity\ of\ alkali}$$

$$Uronic\ acid\ anhydride\% = \frac{molar\ mass\ of\ uronic\ acid\ anhydride \times 100}{acid\ equivalent\ weight}$$

### 3.2.10 Determination of Sugars

Determination of Sugars was done by High Performance Liquid Chromatography (HPLC) technique after acid hydrolysis of habil gum .

#### 3.2.10. 1 Procedure

Aliquots of 0.03 to 0.05 grams of gum were, accurately, weighed into tared stoppered test tubes.Ten mls of 4% sulphuric acid were added to each tube. The tubes were then placed into a boiling water bath for 4 to 7 hours. The tubes were removed from the water bath and allowed to cool to room temperature. Hydrolysis products were neutralized by the addition of 2 grams of barium carbonate to each tube until the effervescence of $CO_2$ stops. The tubes were allowed to stand in a shaker overnight, and finally filtered. HPLC column was flushed with the mobile phase, acetonitrile : water mixture (80:20 v/v), at a flow rate of 0.8 $cm^3$ / minute till a stable base line is obtained. The hydrolysate of gum sample, which was filtered in line, was injected into a 60 microliter loop of the injection valve of the HPLC system. The injection valve was turned to allow for eluting the hydrolysate through the 25x0.46 cm carbohydrate column. Retention times of monosacchride components were recorded using adifferential refractometer. The proportion of each monosaccharide can be determined from a standard curve prepared using standard sugars. Concentration of sugars, in the gum samples, was estimated from a peak hight – concentration curve constructed using standard sugars (Phillips, *et al* 1989).

### 3.2.11 FTIR- spectra and functional group elucidation

Fourier Transform Infrared Spectrophotometer (IRPrestige-21, Shimadzu, Japan) was used to obtain infrared spectrum of *Combretum glutinosum* gums samples. KBr powder was shattered into fine particles in the agate mortar. Next equalized the grain size by furnished filter. Heated and dried the powder and the sample of habil gum at 120º C~150ºC for more than 24hours and kept it in desiccators. The quantity of KBr powder which was necessary for one forming is 200mg. That of the sample was about 0.5% ~ 1% of KBr powder. 2mg ~ 4mg of sample were

added to 400mg of KBr powder, mixed well in the agate mortar and 200mg were taken for forming. Mixed powder of KBr was put carefully into concave of the tablet frame, on the sample base with the spatula to make KBr tablet by hand press system. Pressing was made while evacuation was being applied. The working pressure was 78.5KN (8ton). the pressing time was 5 ~ 10min. After pressing KBr tablet were taken out and mounted on the tablet holder then inserted into the window of the sample compartment, and a measurement was made as background. Steps above were repeated for sample tablet, then the spectrum was produced. (Manual of Fourier Transform Infrared Spectrophotometer IRPrestige-21)

### 3.2.12 Determination of number average molecular weight (Mn)

One of the precise techniques of macromolecules molecular weight determination involves the measurement of osmotic pressure. The instrument for carrying out these measurements is called an Osmometer. The device consists of a semipermeable membrane that separates two solution compartments. The semipermeable membrane is made of a material that permeates the solvent (not the solute). If the membrane separates pure solvent from a solution, an osmotic pressure develops across the membrane which, in turn, drives the flow of solvent through the membrane from the pure solvent compartment to the solution compartment. The flow of solvent that occurs due to a concentration gradient across the membrane is called osmosis.

Osmotic pressure is a colligative property, which means that it is proportional to the concentration of solute. The van't Hoff equation is often presented in introductory chemistry for calculating osmotic pressure (Π) from the number of moles of solute ($n_{solute}$) that occupy a given volume (V) and the absolute temperature (T) of the solution

$$\Pi = \frac{n_{solute} RT}{V} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

There is similarity between equation (1) and the ideal gas equation ($P=nRT/V$). But just like the ideal gas equation, the van't Hoff equation is only valid for an

ideal system. In this case, equation (1) is only valid for an ideal solution, which is a hypothetical solution in which the solute-solvent, solvent-solvent, and solute-solute interactions are all equivalent. Since all non-volatile, non-electrolytic solutions approach ideal behavior in the dilute limit, equation (1) is actually a limiting law, and could be written in the form

$$\Pi = \quad \lim_{n_{solute \to 0}} \left(\frac{nRT}{V}\right) \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

When using an osmometer, it is more convenient to express concentration in terms of 'grams' of solute per liter

$$C = \frac{g}{l}$$

Whereby one can make the following substitution in equation (2)

$$\frac{n_{solute}}{V} = \frac{C}{M}$$

Where M is the molecular weight of the solute. And with minor rearrangement, equation (2) can be written as

$$\lim_{c \to 0} \frac{\Pi}{C} = \frac{RT}{M} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

According to equation (3), the molecular weight of a solute can be obtained by plotting specific osmotic pressure versus concentration and extrapolating linear curve to obtain its intercept.

Since equation (3) is only exact in the dilute limit, one can recognize this relationship as the first term in a more general power series expansion in *c*

$$\frac{\Pi}{C} = RT\left[\frac{1}{M} + A_2C + A_3C^2 + \cdots\right] \ldots\ldots (4)$$

Where $A_2$ and $A_3$ are called the second and third virial coefficients, respectively. These coefficients are empirically determined constants for a given solute-solvent system, and also depend on temperature.

According to statistical mechanical solution theory, $A_2$ represents the interaction of a single solute particle with the solvent, and higher order virial coefficients are associated with correspondingly larger number solute particle cluster interactions with the solvent (Sun, 2004).

**3.2.12.1 Procedure**

The osmotic pressure of gum was determined for a series of aqueous solution of different concentrations using Osmomat 050 Colloidal Osmometer (Genotech, germany) at 21˚C. The $\frac{\Pi}{C}$ were plotted versus concentration and the intercept of the curve was determing

**3.2.13 Viscosity**

**3.2.13.1 Procedure**

Viscosity was determined using Ubbelohde viscometer ASTM-IP (size 1C). Gum samples were dissolved in 1M NaCl solution to give solution with concentration of 4%. The solutions were filtered through whatman filter paper No. (42) in to clean container.

The viscometer was cleaned by washing with distilled water, sodium chloride solution and dried in acetone. Exactly 14ml of filtrate were pipetted into the reservoir of the viscometer contained in water path set at 25 ˚C. The efflux time was then measured for the original solution and six of its dilution (adding 1m for each dilution). The average of three measurements was taken for each concentration. The efflux time of the solvent (1M NaCl) was then measured.

**Relative viscosity determination ($\eta_r$)**

In study of dilute polymer solutions, instead of absolute value of the viscosity coefficient one often uses the relative viscosity which is defined as the ratio of solution viscosity $\eta_{sol}$ to pure solvent viscosity $\eta_{solv}$

$$\eta_r = \frac{\eta_{sol}}{\eta_{solv}} \quad \text{……………………. (1)}$$

To determine relative viscosity, the time of efflux of the solution and solvent are measured in the same viscometer, taking the density of the dilute solution equal to that of the pure solvent. ($d=d_o$) as the solution is highly diluted.

$$\eta_r = \frac{t}{t_o} \text{ ..........................} (2)$$

The efflux times of the solution t and the solvent $t_o$ are determined at strictly fixed temperature, as viscosity depends on temperature.

**Specific viscosity ($\eta_{sp}$)**

Specific viscosity is the viscosity of the solution upon the addition of the polymer (fractional change), in other words it is the viscosity increase due to the presence of a polymer relative to the viscosity of the pure solvent.

$$\eta_{sp} = \frac{\eta_{sol} - \eta_{solv}}{\eta_{solv}} \text{.................} (3)$$

$$\eta_{sp} = \eta_r - 1 \text{.........} (4)$$

**Reduced viscosity**

Reduced viscosity is the fractional change in viscosity per unit of polymer concentration or it is the ratio of the specific viscosity to the concentration.

$$\eta_{red} = \frac{\eta_{sp}}{C} \text{..........................} (5)$$

Intrinsic viscosity is the limit of the reduced viscosity as the polymer concentration (C) approaches zero.

The relative, specific and reduced viscosities of dilute solutions increase with increase in concentration. The variation of reduce viscosity with concentration in dilute solution is given by a straight line described by equation.

$$\frac{\eta_{sp}}{C} = a_1 + a_2 c \text{.....................} (6)$$

Where $a_1$ = intercept on ordinate

$a_2$ = slope of line

The quantity

$$a_1 = lim \left[\frac{\eta_{sp}}{C}\right]_{c \to 0} \text{.................} (7)$$

Which is independent of the concentration of solution and is the limiting value of

the reduced viscosity, is known as the limiting viscosity number (intrinsic viscosity [η]) of the solution ;

$$[\eta] = lim\left[\frac{\eta_{sp}}{C}\right]_{c\to 0} \quad \ldots\ldots\ldots\ldots\ldots.. (8)$$

Substituting equation (8) into (6) we get

$$\frac{\eta_{sp}}{C} = [\eta] + a_2 c \ldots\ldots\ldots\ldots\ldots\ldots\ldots (9)$$

(Abdelrahman 2008)

**3.2.13.2 Intrinsic viscosity**

The determination of the intrinsic viscosity of polymer solutions is a good alternative to monitor the variation of polymer average molecular weight during polymerization reactions. This is especially true for polymer resins that present poor solubility in the organic solvents that are frequently used for size exclusion chromatography (SEC) and light scattering measurements. The intrinsic viscosity can be related to the polymer average molecular weight through the well-known Mark–Houwink equation, $[\eta] = K \times M^a$ .

Where $M^a$ = Average molecular weight.

[η] = Intrinsic viscosity

K and a = Mark – Houwink constants

The intrinsic viscosity also can be determined experimentally through measurements of the inherent and specific polymer viscosities, (Chamberlain and Rao, **2000**). It is usually determined as the limit of the inherent viscosity or of the ratio between the specific viscosity and the polymer concentration when the polymer concentration approaches zero.

$[\eta] = \lim_{c\to 0}[\eta inh] = \lim_{c\to 0}[\eta red]$

Where

ηinh = Inherent viscosity.

ηred = Reduced viscosity.

### 3.3 Cytotoxicity methods

In this study, a cytotoxicity method was used to investigate the toxicity of the *Combretum glutinosum* samples collected from Blue Nile, (Eldamazaein area), using different types of normal and cancer human cell lines.

### 3.3.1Materials and chemicals

#### 3.3.1.1 Materials

A representative composite sample of each season 2007, 2008 was used to evaluate the toxicity of *Combretum glutinosum* gum.

#### 3.3.1.2 Cell lines

Four cell lines namely human normal melanocytes cell line (HFB4), baby hamster normal kidney fibroblast cell line (BHK), human hepatocellular carcinoma cell line (HEPG2) and human colon carcinoma cell line (HCT116) were obtained frozen in liquid nitrogen from the American Type Culture Collection (ATCC, Manassas, USA) and were maintained by serial sub-culturing in the National Cancer Institute, Cairo, Egypt. The cell lines were grown as "monolayer culture" in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 2 ml streptomycin.

#### 3.3.1.3 Chemical and solutions

The following chemicals and solutions were used:

1- Dimethylsulphoxide (DMSO) (Sigma-Aldrich Chemical Co., ST. Louis, MO, U.S.A.): It was used for cryopreservation of cells.

2- RPMI-1640 medium (Sigma-Aldrich Chemical Co., ST. Louis, MO, U.S.A.): The medium was used for culturing and maintenance of the cell lines. The medium was supplied as a powder form. It was prepared as follows: 10.4 gm medium was weighed, mixed with 2 gm sodium bicarbonate, completed to 1 L with distilled water and shacked carefully till complete dissolution. The medium was then sterilized using a Millipore bacterial filter (0.22µm). The prepared medium was kept in a refrigerator (4 $^{0}$C) and checked at regular intervals for contamination.

Before use, the medium was warmed at 37 $^0$C in a water bath after the addition of penicillin/streptomycin and 10 % fetal bovine serum (FBS).

RPMI-1640 was developed by Moore et al. at Roswell Park Memorial Institute, hence the acronym RPMI. RPMI 1640 is a basal medium consisting of vitamins, amino acids, salts, glucose, glutathione and a pH indicator. It contains no proteins or growth promoting agents. Therefore, it requires supplementation to be a “complete” medium. It is most commonly supplemented with 5-10% Fetal Bovine Serum (FBS). RPMI 1640 utilizes a sodium bicarbonate buffer system (2.0 g/L) and therefore requires 5% $CO_2$ to maintain the required pH. When exposed to ambient levels of $CO_2$, the sodium bicarbonate in the medium will cause RPMI to become very rapidly basic, and a purple color is observed, indicating a rise in pH. Powder media is formulated without sodium bicarbonate because it tends to gas off in the powdered state.

3-Sodium bicarbonate (Sigma-Aldrich Chemical Co., ST. Louis, MO, U.S.A.): It was used for the preparation of RPMI-1640 medium.

4- Fetal Bovine Serum (FBS) (Sigma-Aldrich Chemical Co., ST. Louis, MO, U.S.A.): 10 % FBS (heat inactivated at 56 $^0$C for 30 min) was used for the supplementation of RPMI-1640 medium prior to use.

5- Penicillin/Streptomycin (Sigma-Aldrich Chemical Co., ST. Louis, MO, U.S.A.): 100 units/ml Penicillin and 2 mg/ml Streptomycin were used for the supplementation of RPMI-1640 medium prior to use.

Glutamine (Sigma-Aldrich Co., St. Louis, MO, USA). It was added to RPMI-1640 medium. The concentration used was 300 mg/liter.

6- Trypan blue dye: A stock solution was prepared by dissolving one gram of trypan blue (Sigma- Aldrich Co., St Louis, MO, USA) in 100 ml saline. The working solution was prepared by diluting one ml of stock solution with nine ml of saline. The dye was used for staining the dead cells for viability counting.

7- MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide] was obtained from Sigma Chemical Company (St. Louis, MO). It was prepared as 5mg/ml of MTT in 0.9% NaCl, used in cytotoxicity assay.

8- Isopropanol/ HCl: It was used as acidified solution contains (0.04 N HCl in absolute isopropanol) in the MTT assay in the *in vitro* study. It was obtained from (Lab Scan Analytical Science Co., Dublin, Ireland).

9- Sodium Dodecyl Sulfate (SDS) was obtained from Sigma-Aldrich Chemical Co., ST. Louis, MO, U.S.A.: 100 µl of 10% (SDS) in 0.01 N HCl.

10- Hydrochloric acid (HCl): was obtained from (United Company for Chem. & Med. Prep., Cairo, Egypt).

### 3.3.2 MTT assay

Cytotoxicity of different aqueous gum concentrations was determined using the MTT method (Mossman, 1983).Figure3.3. The method is based on the ability of active mitochondrial dehydrogenase enzyme of the living cells to cleave the tetrazolium rings of the yellow MTT and form dark blue insoluble formazan crystals which cannot pass the cell membranes, resulting in its accumulation within healthy cells. Solubilization of the cells results in the liberation of crystals, which are then solubilized. The number of viable cells is directly proportional to the intensity of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm. This conversion does not take place in dead cells.

The MTT method proved to be useful to estimate cell densities in small culture volumes and was more accurate and reliable than hemocytometer counting. The cultivation in small culture volumes and, the sensitive, evaluation with the MTT assay allow the screening and testing of many different substances, fractions, and nutrients indispensable to the development of defined media for the cultivation of such cells (Freimoser et al 1999).

### 3.3.3 Maintenance of the cell lines in the laboratory*:*

A cryotube containing frozen cells was taken out of liquid nitrogen container and thawed in a water bath at 37$^{o}$C. It was opened under strict aseptic conditions and its contents were supplied by 5 mls supplemented medium drop by drop in a 50 ml disposable sterile falcon tube. The tube was incubated for 2 hrs then its contents were centrifuged at 1200 rpm for 10 minutes to discard the preserving DMSO. The cell pellet was suspended and seeded in 5 ml supplemented medium in T25 Nucleon sterile tissue culture flasks. The cell suspension was incubated and followed up daily with replacing the supplemented medium every 2-3 days. Incubation was continued until a confluent growth was achieved and the cells were freshly subcultured.

### 3.3. 4 Collection of cells by trypsinization:

The medium was discarded and cells were washed twice with 5ml phosphate buffered saline. All the adherent cells were dispersed from their monolayer by the addition of 1 ml trypsin solution (0.025 % trypsin w/v).The flask was left in the incubator till complete detachment of all the cells and checked with the inverted microscope (Olympus 1x70, Tokyo, Japan).Trypsin was inactivated by the addition of 5 ml of the supplemented medium containing FBS. The supernatant was discarded by centrifugation at 1200 rpm for 10 minutes .The pellet was collected and separated into single cell suspension by gentle dispersion several times.

### 3.3.5 Determination and counting of viable cells:

50 µl of 0.05 % trypan blue solution was added to 50 µl of the single cell suspension. The cells were examined and counted under the inverted microscope using the haemocytometer. Non stained (viable) cells were counted and the following equation was used to calculate the cell count /ml of cell suspension.

$$\text{Viable cells/ml} = \frac{\text{number of cells in 4 quarters x 2 (dilution factor) x } 10^4}{4}$$

The cells were then diluted to give the required cell number for each experiment.

**3.3.6 Cryopreservation of cells:**

To avoid loss of the cell line, excess cells were preserved in liquid nitrogen as follows:

Equal parts of the cell suspension and freezing medium (10% DMSO in supplemented medium) were dispersed into cryotubes. It was racked in appropriately labeled polystyrene boxes, gradually cooled till reaching -80 $^oC$.

Then the cryotubes were transferred to a liquid nitrogen (-180 $^oC$) till their use.

**3.3.7 Procedure**

Cells were seeded in 96-well microtiter plates at a concentration of $5x10^3$ cells / well in a 150µl fresh medium and incubated for 24 hrs at 37°C in 5% $CO_2$-95% air in a high humidity atmosphere in the water jacketed incubator (Revco, GS laboratory equipment, RCO 3000 TVBB, USA.). After 24 hrs, 50µl of different concentrations of gum solutions (250, 500, 750, 1000 µg mL-1) were added; in addition control cells treated with vehicles alone were also included. Cell free culture medium served as the negative control, and phenol was included as the positive control. Incubation was continued for 24, 48 and 72 hrs. After incubation, 10 µl of MTT stock (5 mg/ml of MTT in saline) was added to each well. After incubation for 4 h, 100 µl of 10% Sodium Dodecyl Sulfate (SDS) in 0.01 N HCl was added to each well to terminate the reaction. The solution was allowed to solubilize overnight in 100% humidity. Then, photometric determination of the absorbance was achieved at 570 nm using micro plate Enzyme Linked Immunosorbent Assay (ELISA) reader (Meter tech. Σ 960, USA). Triplicate repeats were prepared for each concentration and the average was calculated. The experiment was repeated three times independently (Zang *et al.* 2003).This procedure was made for two samples from two seasons of *Combretum glutiosum* gum. Data were expressed as the percentage of relative viability compared with the control group (cells suspended in RPMI media without gum).

Calculation: The percentage of cell survival was calculated as follows:

$$\textbf{\% cell survival} = \frac{T}{C} \times 100$$

Where: T is the number of viable cells in unit volume of the test drug well.

C is the number of viable cells in unit volume of the control well.

Survival fraction = Optical density (treated cells)/ Optical density (Control cells).

The $IC_{50}$ were calculated from the survival curve of different concentrations using (Table curve TM) for windows (Jandel scientific, 1992). Triplicate experiments were conducted for each cell line.

**Figure 3.2: Chemical structure of a dimethyl sulfoxide (DMSO) molecule**

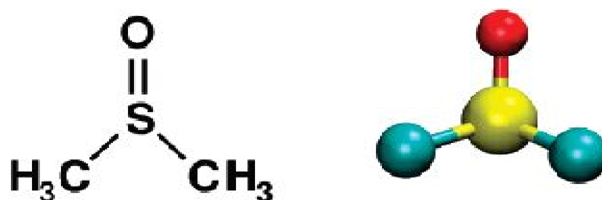



**Figure 3.3: Molecular Structure of MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5- diphenyltetrazolium bromide)**

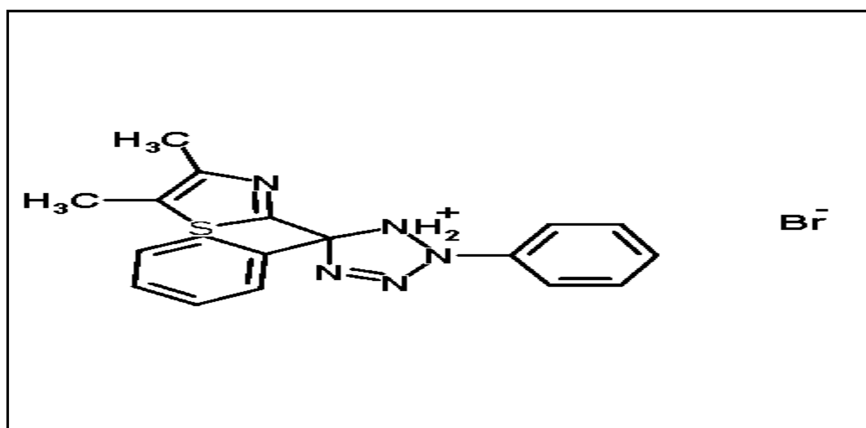


**Figure [3.4]: cryotubes**

**Figure [3.5]: T25 Nucleon tissue culture flasks**

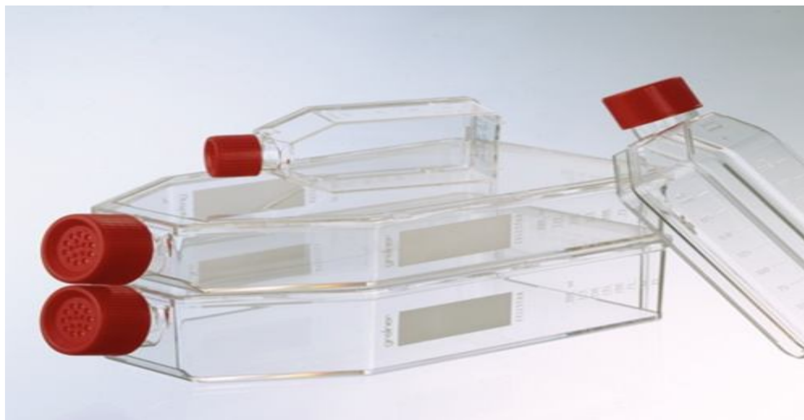


**Figure [3.6]: Falcon tube**

# Chapter Four

## Results and Discussion

### 4.1 Physicochemical characterization results

Chemical and physical properties of gum are considered as important characters for the article of commerce, to ensure the identity and purity of gum and avoiding mixing and adulteration.

#### 4.1.1 Physicochemical results

Tables 4.1, shows the analytical data of physicochemical properties for twenty samples of *Combretum glutinosum* gum. The moisture content, of nodule samples, was in the range 6.7 to 9.9 % with the mean value of 7.96%, the ash content was found to fall in the range of 1.84 to 6.39% with the mean value of 4.51 %. Nitrogen content was found to be in the range 0.18 to 0.88% with the mean value of 0.37%, and the protein content was calculated from the Nitrogen content, using Nitrogen conversion factor (6.6), and it falls in the range 0.946 to 5.861% with the mean value of 2.43%. Tannin, starch and dextrin in *Combretum glutinosum* gums were not detected at any level.

#### 4.1.2 Cationic composition of *Combretum glutinosum* samples

Tables 4.2 and 4.3 show that calcium, magnesium, potassium and sodium are the most abundant element in *Combretum glutinosum* gum with the mean values 31.20, 24.17, 13.16 and 0.28 respectively. This indicate that *Combretum glutinosum* gum is a salts of calcium, magnesium, and potassium. While Table 3.5 shows the quantity of iron, cobalt, nickel, cadmium, lead, copper, zinc, chromium and aluminium recorded as p.p.m, with the mean values 0.55, 0.065, 0.2, 0.163, 0.064, 0.015, 0.064, 2.07and 1.272 respectively, these cations were found in trace amounts in habil gum.

### Table 4.1 Analytical data of physicochemical analysis of *combretum glutinosum* gum

| | Samples of *Combretum glutiosum* | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **parameter** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **1** |
| **Moisture content%** | 7.74 | 9.86 | 7.89 | 7.02 | 7.33 | 7.04 | 7.71 | 7.55 | 8.09 | 7.52 | 6.70 | 7.54 | 8.44 | 9.20 | 9.31 | 8.76 | 7 |
| **Ash%** | 4.10 | 5.99 | 3.50 | 4.68 | 3.54 | 1.84 | 4.54 | 3.84 | 4.44 | 3.94 | 3.84 | 5.69 | 5.14 | 4.24 | 4.64 | 4.9 | 5 |
| **pH** | 4.49 | 5.49 | 5.50 | 3.81 | 4.03 | 3.88 | 4.66 | 4.35 | 4.29 | 4.49 | 4.97 | 4.60 | 4.72 | 4.52 | 4.62 | 4.42 | 4 |
| **Nitrogen %** | 0.43 | 0.14 | 0.71 | 0.5 | 0.89 | 0.35 | 0.19 | 0.29 | 0.42 | 0.39 | 0.39 | 0.29 | 0.31 | 0.36 | 0.36 | 0.36 | 0 |
| **Protein%** | 2.84 | 0.95 | 4.70 | 3.30 | 5.86 | 2.31 | 1.25 | 1.96 | 2.31 | 2.57 | 2.61 | 1.92 | 2.06 | 2.40 | 2.37 | 2.35 | 1 |
| **Starch or dextrin** | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | N |
| **Tannin** | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND | N |

***X = Average value**
**** S = Standard deviation**
**ND = Not detected**

### Table 4.2 Cationic composition of gum samples.

| | Samples of *Combretum glutiosum* | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Elements p.p.m** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** |
| **Ca** | 34.83 | 116.5 | 52.1 | 28.7 | 24.42 | 38.17 | 12.81 | 10.72 | 49.1 | 73.1 | 27.6 | 69.56 | 111.3 | 40.47 | 80.39 | 23.5 | 17.4 |
| **K** | 103.1 | 16.1 | 7.36 | 48.3 | 52.68 | 42.12 | 42.62 | 45.90 | 4.12 | 35.7 | 62.6 | 15.55 | 24.17 | 9.90 | 1.38 | 45.5 | 33. |
| **Mg** | 10.38 | 5.019 | 6.77 | 7.14 | 5.91 | 1.76 | 26.84 | 20.63 | 24.3 | 5.71 | 7.02 | 7.80 | 9.72 | 33.52 | 33.34 | 10.2 | 25.4 |

| Na | 1.15 | 0.03 | 0.03 | 0.04 | 0.122 | 0.04 | 0.08 | 0.001 | 0.04 | 0.52 | 0.41 | 0.02 | 0.023 | 0.03 | 0.32 | 0.01 | 0.0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|

***X = Average value**
**** S = Standard deviation**

## Table 4.3 Result of Determination of Heavy Metals

| | **Samples of *Combretum glutiosum*** | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Elements p.p.m** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **17** |
| **Fe** | 0.96 | 0.74 | 0.81 | 0.58 | 0.54 | 0.34 | 0.74 | 0.34 | 0.15 | 0.51 | 0.51 | 0.47 | 0.59 | 0.45 | 0.52 | 0.18 | 0.6 |
| **Ni** | 0.01 | 0.04 | 0.08 | 0.15 | 0.12 | 0.09 | 0.07 | 0.111 | 0.02 | 0.07 | 0.04 | 0.03 | 0.06 | 0.04 | 0.003 | 0.05 | 0.0 |
| **Cd** | 0.018 | 0.001 | 0.01 | 0.01 | 0.012 | 0.004 | 0.002 | 0.012 | 0.33 | 0.32 | 0.35 | 0.33 | 0.33 | 0.32 | 0.33 | 0.3 | 0.2 |
| **pb** | 0.06 | 0.05 | 0.01 | 0.03 | 0.05 | 0.074 | 0.05 | 0.03 | 0.11 | 0.61 | 0.08 | 0.37 | 0.56 | 0.38 | 0.32 | 0.19 | 0.0 |
| **Co** | 0.099 | 0.02 | 0.1 | 0.08 | 0.16 | 0.04 | 0.09 | 0.08 | 0.04 | 0.03 | 0.09 | 0.06 | 0.07 | 0.04 | 0.05 | 0.07 | 0.0 |
| **Cu** | 0.003 | 0.028 | 0.02 | 0.00 | 0.04 | 0.00 | 0.017 | 0.02 | 0.00 | 0.01 | 0.02 | 0.03 | 0.017 | 0.035 | 0.002 | 0.01 | 0.0 |
| **Zn** | 0.053 | 0.082 | 0.04 | 0.03 | 0.04 | 0.11 | 0.07 | 0.09 | 0.04 | 0.08 | 0.06 | 0.09 | 0.07 | 0.06 | 0.07 | 0.04 | 0.0 |
| **Cr** | 1.698 | 1.7 | 0.99 | 1.6 | 2.89 | 2.5 | 2.52 | 2.37 | 2.5 | 1.5 | 2.76 | 3.14 | 1.99 | 1.32 | 1.14 | 2.68 | 1.7 |
| **Al** | 0.98 | ND | 0.76 | 0.92 | 1.90 | 0.37 | 0.92 | 0.09 | 0.90 | 3.23 | 2.00 | ND | 0.079 | 1.016 | 2.37 | ND | 1.9 |

***X = Average value , ** S = Standard deviation, ND = Not detected**

### 4.1.3 Acid equivalent weight and uronic acid content of *Combretum glutinosum*

Table 3.4 shows the apparent equivalent weight and uronic acid content of *Combretum glutinosum* gum samples, the apparent equivalent weight was found to fall within a range of 964.9 to 2083.4% with mean value of 1524.15%.While uronic acid value was found in the range of 9.9 to 21.15 % with a mean value of 15.53%.

**Table 4.4 Acid equivalent weight and uronic acid of *Combretum glutinosum* gum :**

| Analysis | Season 2007 | Season 2008 | Average | Standard average deviation |
|---|---|---|---|---|
| Acid Equivalent weight | 964.9 | 2083.4 | 1524.15 | 790.90 |
| Uronic acid | 21.15 | 9.9 | 15.53 | 7.95 |

### 4.1.4 Solubility of *Combretum glutinosum* gum composite sample

Table 3.5 show the solubility of *Combretum glutinosum* gum in several solvent. It is Low soluble in water its only 30% soluble in a cold water, while it dissolved in bases to larger extent. It dissolved to a level of 40% in EDTA and 96.3% in $Na_2CO_3$ .

**Table 4.5 Solubility of *Combretum glutinosum* gum composite sample**

| Sample / Solvent | Composite 2007 | Composite 2008 |
|---|---|---|
| Water | 58.6% | 30% |
| EDTA | 63.8% | 40% |
| $Na_2CO_3$ | 96.3% | 95.8% |

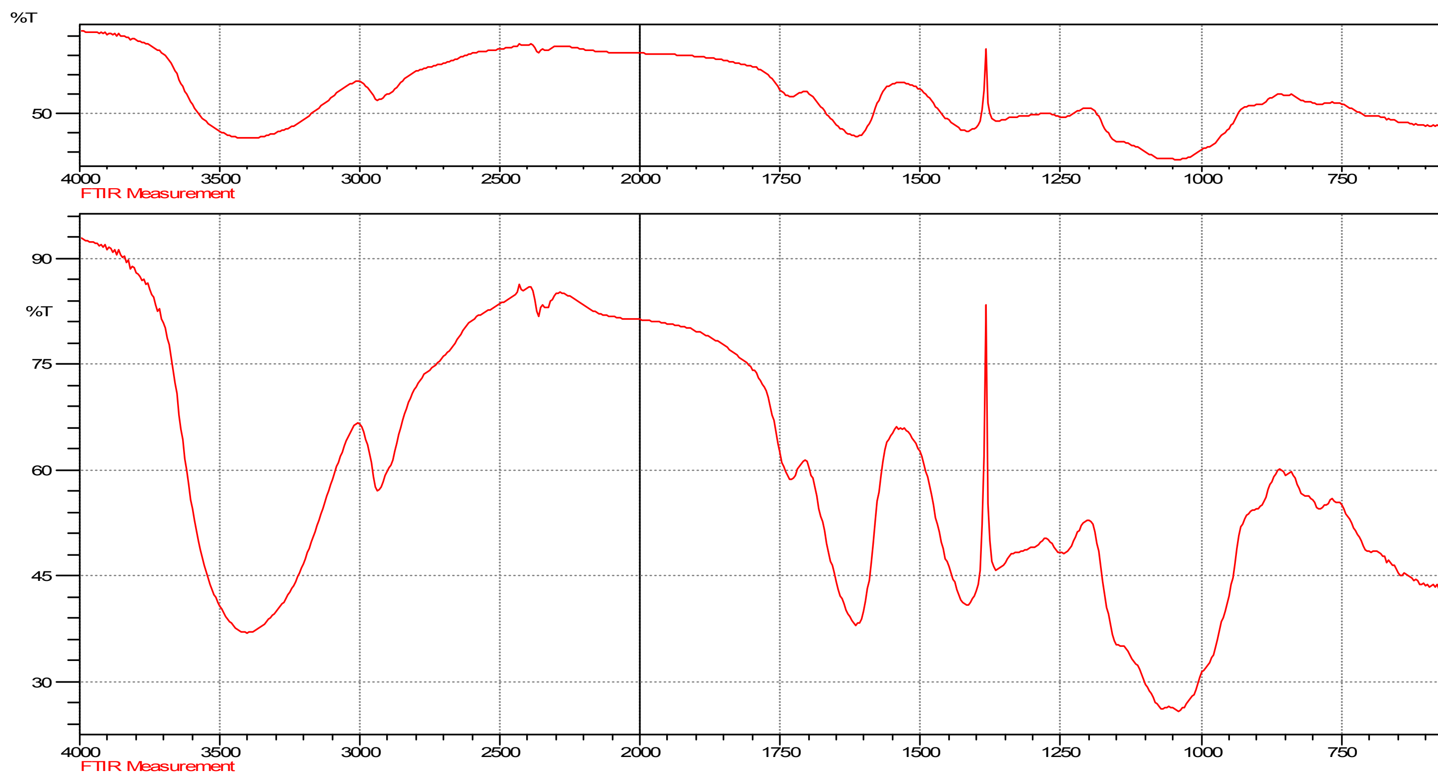
### 4.1.5 FT-IR Spectrum of *Combretum glutinosum* and its fractions

Figures.3.6 A, B and C show the peaks of functional groups frequencies appeared at different wave number ($cm^{-1}$), on FT-IR spectrum of freeze dried soluble fraction of three composite gum samples. All *Combretum glutinosum* gum samples showed the presence of hydroxyl, alkanes, carbonyl, alkenes, and phenols functional groups at 3200-3500, 2850-2999, 1680-1740, 1600-1700, 1375-1450 and 1200 $cm^{-1}$ respectively. FT-IR spectrum of freeze dried soluble part, the gum for season 2007, season 2008 and composite sample of two seasons showed similar functional groups indicating no effective difference due to climate, soil and other environmental factor. (Brito *et al*., **2004**).

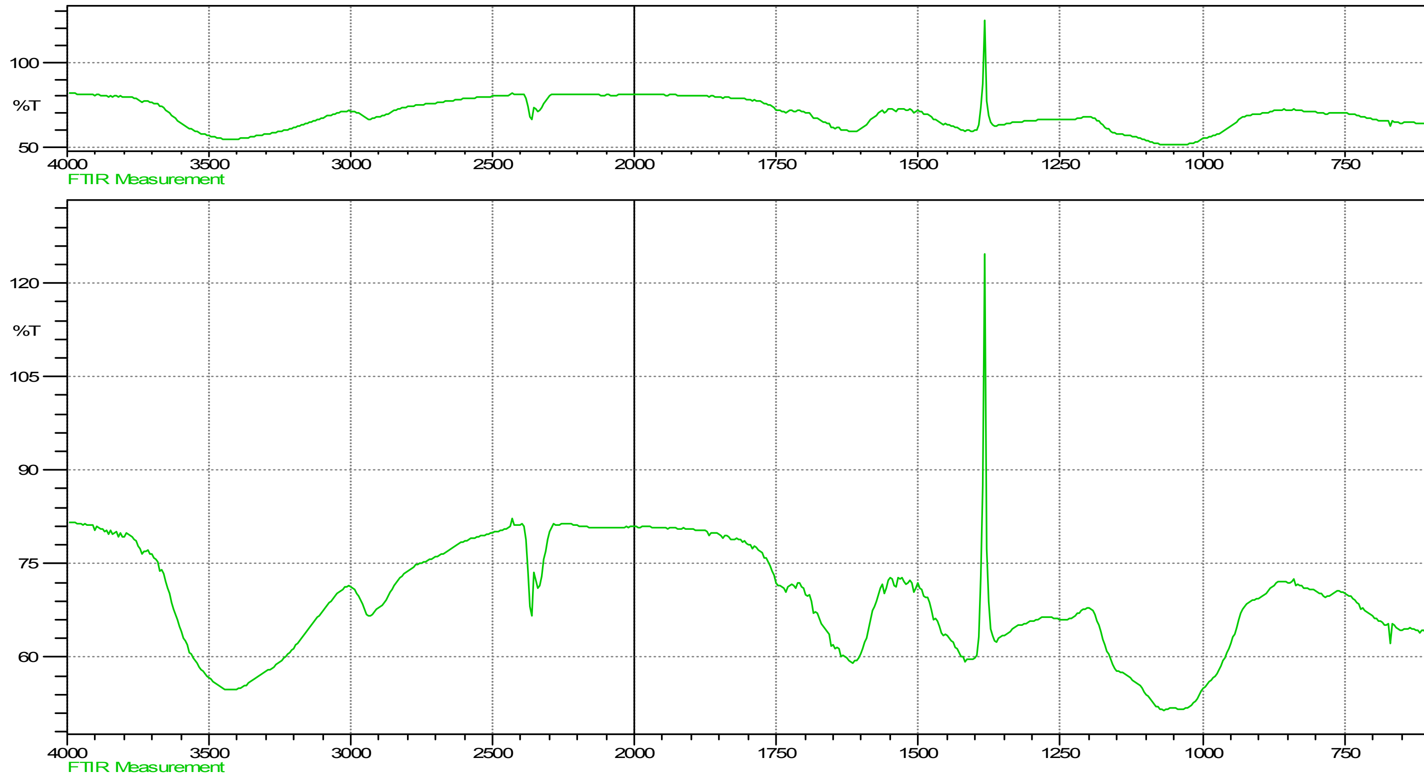
**Table 4.6 FT-IR Spectrum of *Combretum glutinosum* gum**

| Gum sample | Sample absorbance frequencies ($cm^{-1}$) | Bond | Functional group | References absorbance frequencies ($cm^{-1}$) |
|---|---|---|---|---|
| Season 2007(A) | 3420 | O-H | Hydroxyl | 3200-3600 |
| Season 2008 (B) | 3400 | | | |
| Composite A,B | 3420 | | | |
| Season 2007(A) | 2910 | − C-H | Alkanes | 2850-2999 |
| Season 2008 (B) | 2930 | | | |
| Composite A,B | 2930 | | | |
| Season 2007(A) | 1740 | C=O | Carbonyl | 1680-1740 |
| Season 2008 (B) | 1735 | | | |
| Composite A,B | 1740 | | | |
| Season 2007(A) | 1610 | − C=C− | Alkenes | 1600-1700 |
| Season 2008 (B) | 1610 | | | |
| Composite A,B | 1610 | | | |
| Season 2007(A) | 1200 | H<br>C- O | Phenols | 1200 |
| Season 2008 (B) | 1200 | | | |
| Composite A,B | 1200 | | | |

**Figure 4.1 (A) FTIR measurement of *Combretum glutinosum* gum season 2007**




**Figures 4.1 (B) FTIR measurement of *Combretum glutinosum* gum season 2008**




**Figures 4.1 (C) FTIR measurement of *Combretum glutinosum* gum of Composite sample (2007-20**

### 4.1.6 Osmotic pressure and polymer molecular weight

Tables 4.7 and 4.8 show a series of aqueous solution of a freeze dried composite samples of *Combretum glutinosum* seasons 2007 and 2008. Osmotic pressure was determined using Osmomat 050 Colloidal Osmometer at 21˚C.

Figures 4.2, 4.3 show the relationship between osmotic pressure of *Combretum glutiosum* gum Season 2007 and 2008 and concentration out of which molecular weight was calculated using the equation below

$$\frac{\Pi}{C} = RT\left[\frac{1}{M} + A_2C \ldots\right]$$

Where

$A_2$ is the second virial coefficient, $c$ is the concentration of the solute in g/100 ml, R is the gas constant 82.06 (ml. atmosphere /mol. k), T is the temperature in Kelvin and M is the number average molecular weight. π/c is reduced somatic pressure. The plot π/c versus concentration gives linear relationship. Modified equation of Van't Hoff's above and the intercept of the linear plot used to determine molecular weight. The values of number average molecular weight of *Combretum glutinosum* season 2007 and 2008 were $2.4436 \times 10^4$ and $1.2218\times10^4$ Dalton respectively, with the mean value 18327 Dalton. The result showed that molecular weight of composite sample collected in season 2007was higher than that of season 2008.

**Table 4.7 Osmotic pressure concentration of *Combretum glutinosum* gum**

| Conc.% | Conc.g/ml | Conc.g/l | π mm/Hg | π atmosfer | π (atmosfer)/c( g/l) |
|---|---|---|---|---|---|
| 2% | 0.02 | 0.2 | 5.31 | 0.00698 | 0.035 |
| 3% | 0.03 | 0.3 | 11.68 | 0.0153 | 0.051 |
| 4% | 0.04 | 0.4 | 22.21 | 0.0292 | 0.073 |
| 5% | 0.05 | 0.5 | 33.53 | 0.0441 | 0.088 |
| 6% | 0.06 | 0.6 | 46.6 | 0.0613 | 0.102 |
| 7% | 0.07 | 0.7 | 63.7 | 0.0838 | 0.12 |

Intercept = RT / M ------M = 0.082 ×298/0.001 = 24436

Where R = Gas constant 82.06 ml. atmosfer / (mol. k).

T= Temperature in Kelvin.

M = Molecular weight (Dalton ).

C = Concentration of solute of gum g/ml.

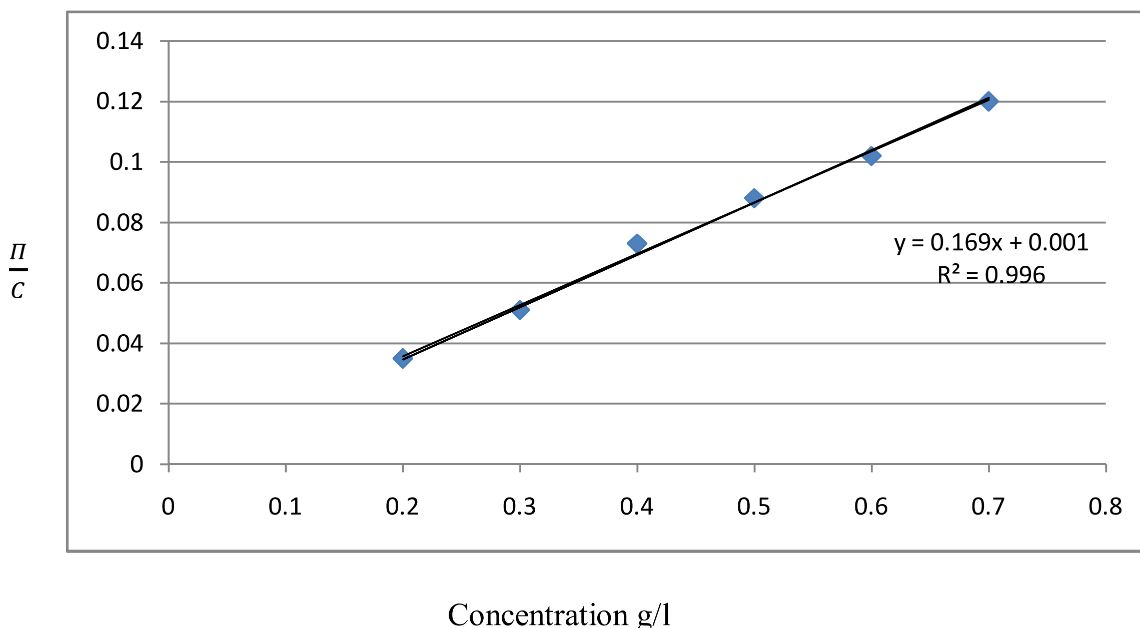



**Figure 4.2: Specific osmotic pressure - concentration relationship for *Combretum glutinosum* gum samples of Season 2007.**

**Table 4.8 Osmotic pressure concentration of *Combretum glutinosum* gum sample collected on Season 2008.**

| Conc.% | Conc.g/ml | Conc.g/l | π mm/Hg | π atmosfer | π (atmosfer)/c (g/l) |
|---|---|---|---|---|---|
| 2% | 0.02 | 0.2 | 6 | 0.0079 | 0.039 |
| 3% | 0.03 | 0.3 | 13.5 | 0.018 | 0.059 |
| 4% | 0.04 | 0.4 | 23.9 | 0.0314 | 0.079 |
| 5% | 0.05 | 0.5 | 36.1 | 0.0475 | 0.095 |
| 6% | 0.06 | 0.6 | 55.1 | 0.073 | 0.121 |
| 7% | 0.07 | 0.7 | 69.8 | 0.092 | 0.131 |

Intercept = RT/M … M = 0.082 ×298/0.002 = 12218

Where R = Gas constant 82.06 ml. atmosfer / (mol. k).

T = Temperature in Kelvin.

M = Molecular weight (Dalton).

C = Concentration of solute of gum g/ml.

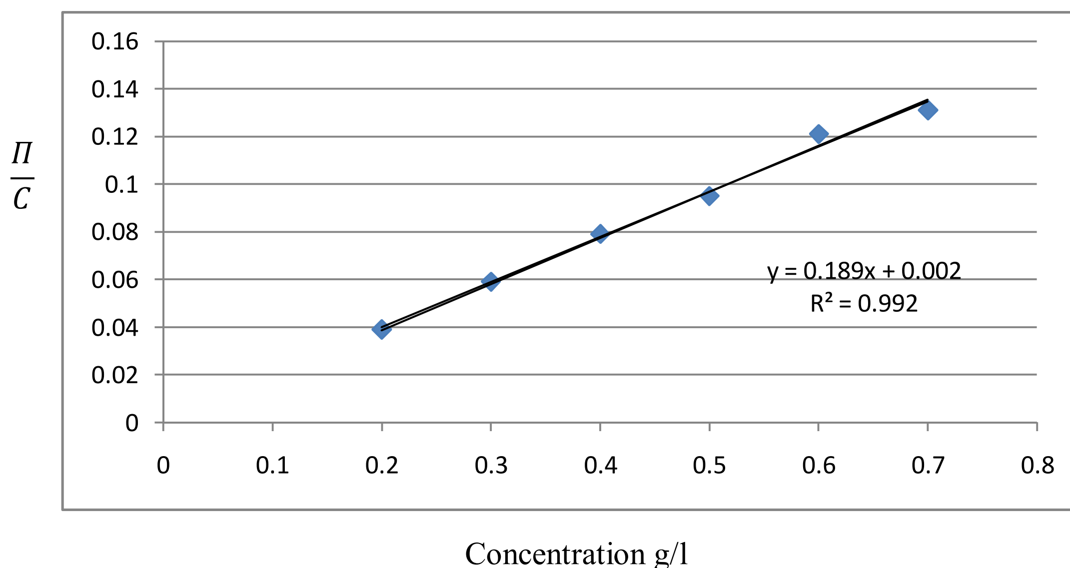



**Figure 4.3: Specific osmotic pressure -concentration relationship for *Combretum glutinosum* gum samples of Season 2008**

### 4.1.7 Determination of sugars

Determination of of Sugars was done by High Performance Liquid Chromatography (HPLC) technique after acid hydrolysis of the gum. Standard calibration curves of sugars were prepared and used to determine unknown concentrations of sugars in gum samples . The concentration of each sugar was calculated from peak area and expressed as a percentage of the total sugar content . The mean value of rhamnose, arbinose and galactose.Were 10.9%, 56.1% and 33% respectively.

**Table 4.9 Sugar % of *Combretum glutinosum* gum**

| **Sugar%** | **Season 2007** | **Season 2008** | **Mean Sugar %** |
|---|---|---|---|
| **Rhamnose** | 10.6% | 11.15% | 10.9% |
| **Arbinose** | 57.9% | 54.2% | 56.1% |
| **Galactose** | 31.4% | 34.5% | 33% |

### 4.1.8 Viscosity

Viscosity was determined using Ubbelohde viscometer, and the method of dilute solution viscosimetry was used to determined intrinsic viscosity and molecular weight. This method based on the measurements of the increase in viscosity of a dilute polymer solution (*Combretum glutinosum* ) and allows to determine the ability of a polymer to increase the viscosity of a particular solvent (NaCl) at a given temperature (25ºC). The intrinsic viscosity was used as a relative method to characterize polymers.

The value of intrinsic viscosity of *Combretum glutinosum* to one composite sample of two seasons 2007and 2008 was11.2 ml $g^{-1}$. This value has been calculated from Table (4.10) which showed the original solution of gum and six of its dilution (adding 1 ml for each dilution), and figure (4.4) showed that the values of $\eta sp$ /C solutions of composite sample when plotted versus gum concentration give linear relationship in low concentration of sodium chloride, or inherent viscosity (the ratio between the specific viscosity and the gum concentration) when the gum concentration approaches zero.

**Table 4.10 Viscosity (η) of *Combretum glutinosum* (composite sample)**

| Conc.% | T(second) | t/t°(Relative) Viscosity | t/t°-1 Specific Viscosity | Sp/c Reduced Viscosity | Lin t/t° ÷c Inherent Viscosity |
|---|---|---|---|---|---|
| 1 | 291.9 | 2.54 | 1.54 | 1.54 | 0.93 |
| 0.89 | 271.03 | 2.35 | 1.35 | 1.51 | 0.956 |
| 0.77 | 240.43 | 2.09 | 1.09 | 1.405 | 0.949 |
| 0.63 | 213.78 | 1.86 | 0.86 | 1.353 | 0.976 |
| 0.46 | 184.01 | 1.60 | 0.60 | 1.29 | 1.0097 |
| 0.33 | 163.155 | 1.42 | 0.42 | 1.26 | 1.05 |

**t°= time of solvent (NaCl) =114.87second.**

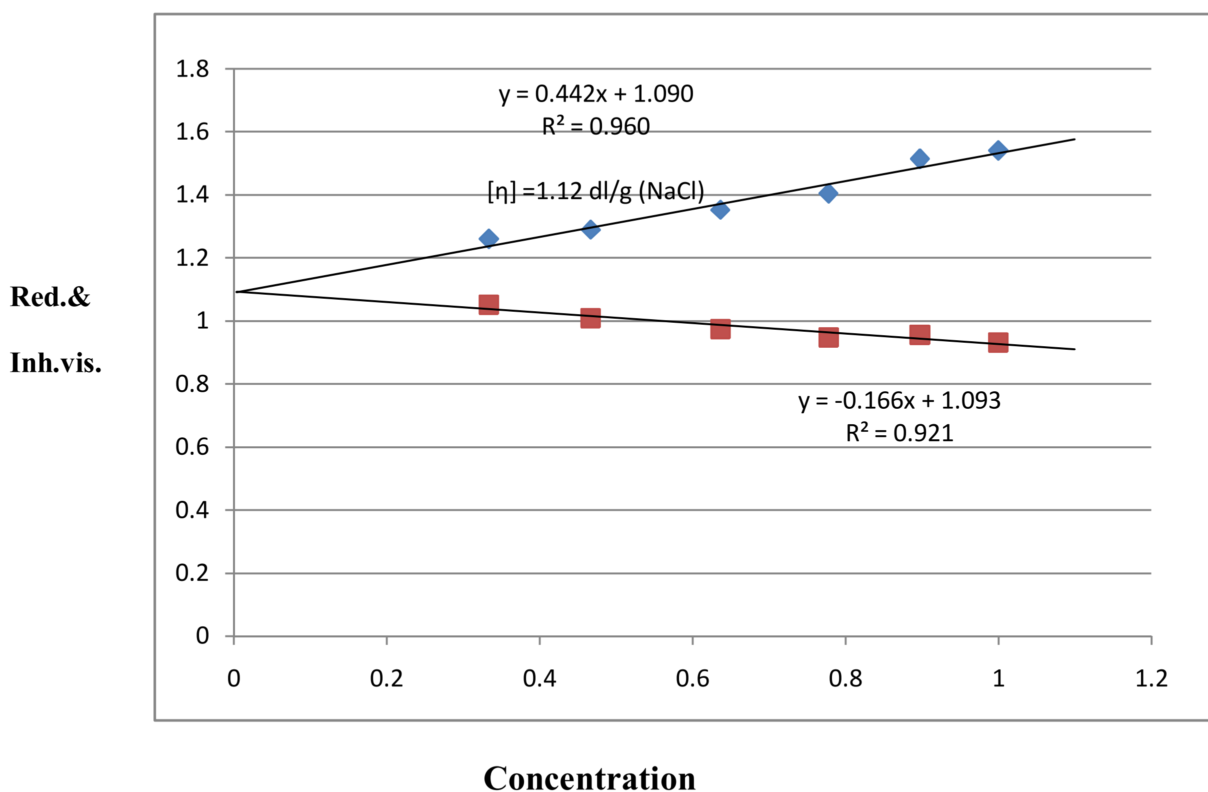



**Figure 4.4 Reduced and inherent viscosity versus concentration of gum solution .**

### 4.2 Characterization discussion

In comparison with the *Acacia* genus where considerable work has been carried out in the field of chemical taxonomy, relatively little is known about gum *Combretum* genus. *Acacias* provided the bulk of the world's commercial supply of gum Arabic. The *Combretum* forests of Africa were not affected to such a great extent, and consequently there has been a great deal of industrial interest in possible utilization of *Combretum* gum, it has local technological applications, but are not recommended for addition to food stuffs.

The botanical sections Metallicum, Glabripetala, Ciliapetala an Angustimarginata are all placed within the sub- genus *Combretum* of the genus*Combretum* Leofl. *Combretum glutinosum* , *Combretum fragrans,*and *Combretum hartmannianum* is placed, together with section Glabripetala. (Exell and Stace 1966).

Table 4.11 showed analytical data for *Combretum glutinosum* gum seasons 2007/2008 compared to *Acacia senegal* 1994/1995. (Karamalla *et al* 1998).

The Moisture content gives an indicator for both purity and real weight, it depend on the season of collecting the samples, and condition of storage. The mean value of moisture content of *Acacia senegal var.senegal* was 10.75 % while for *Combretum glutinosum* 7.96%, this value is not much different from that reported 9.88 (Mubasher 2011). The mean value of pH of *Acacia senegal var.senegal* was 4.40, while the mean value of *Combretum glutinosum* was 4.55. This value agrees with published results 4.79 (Mubasher 2011).The mean value of ash% of *Acacia senegal var.senegal* was 3.77 but the mean value of *Combretum glutinosum* 4.51, which is almost similar to the one reported by Mubasher (4.31). Tannin, Starch and dextrin were not detected in any gum samples of the two species as well as those reported. (Mubasher 2011). *Acacia senegal* gum is highly soluble in water in contrast to *Combretum glutinosum* which shows low solubility in water. It is only 30% soluble in cold water, but it dissolve in bases to larger

extent, it dissolves to a level of 40% in EDTA and 96.3% in $Na_2CO_3$ . These observation were also cited by pervious published results ( Mubasher 2011) .

**Table 4.11: Analytical data for *Combretum glutinosum* gum 2007/2008 compared to *Acacia senegal* 1994/1995.**

| parameter | *Combretum glutinosum (in this study)* | *Combretum glutinosum* (Mubasher 2011) | *Acacia senegal** |
|---|---|---|---|
| Moisture content% | 7.96 | 9.88 | 10.75 |
| pH | 4.55 | 4.79 | - |
| Ash% | 4.51 | 4.31 | 3.77 |
| Nitrogen % | 0.37 | 0.2 | 0.33 |
| Protein% | 2.43 | 1.31 | 2.1 |
| [α]D in Water, degrees | - | - | -31.3 |
| Intrinsic Viscosity, [η] ml/g | 11.2 | 9.80 | 16 |
| Molecular Weight $M_w$ | $2.4 X 10^4$ to $1.2 X 10^4$** | $2.06 X 10^6$ to $9.03 X 10^5$ | $9×10^5$ |
| Acid equivalent weight | 1524.15 | 1291.71 | 1436 |
| Uronic acid | 15.52 | 15.54 | 13.71 |
| Tannin | ND | ND | ND |
| Sugar composition after hydrolysis (%) | | | |
| 4-O- Methylglucuronic acid | - | - | 1.5 |
| Glucuronic acid | - | - | 16 |
| Galactose | 33 | - | 44 |
| Arabinose | 56.1 | - | 25 |
| Rhamnose | 10.9%, | - | 14 |

* (Karamalla *et al* 1998).
** Mn Number average Molecular Weight
ND = Not detect

In this study no appreciable difference between content of nitrogen% and protein% in the two species, the mean of nitrogen% and protein% of *Acacia senegal var.senegal* was 0.33 and 2.1respectively and the mean value of nitrogen% and protein% of *Combretum glutinosum* was 0.37 and 2.43 respectively this mean values is slightly higher than those reported (0.2, 1.31). (Mubasher 2011). The aqueous solution of *Acacia senegal var.senegal* gum having negative Optical rotation of mean value -31.3, but solutions of *Combretum glutinosum* gums were low solubility in water and have unclear solutions that prevent measurement of specific optical rotation.

The mean value of uronic acid content of *Combretum glutinosum* was 15.52,this value is similar to that reported in the literature (Mubasher, 2011) . The mean value of *Acacia senegal* was 13.71. The mean value of acid equivalent weight of *Combretum glutinosum* 1524.15 this value is higher than that reported 1291.71 (Mubasher2011). And slightly higher than value of acid equivalent weight of *Acacia senegal var.senegal* ( 1436). (Karamalla *et al* 1998).

Determination of monosaccharides after acid hydrolysis of the gum samples show that rhamnose was10.9%, arbinose was 56.1% and galactose was 33% in the samples studies. But in *Acacia senegal* in addition to those sugar( rhamnose14%, arbinose 25 % and galactose 44% ) glucuronic acid16%, 4-o-methlglucuronic acid 1.5% was present (Anderson, Bell et al 1977). Therefore monosaccharides rhamnose ,arbinose and galactose were present in both species but with different proportions. The mean value of number average molecular weight from osmotic pressure of *Combretum glutinosum* was$1.8\times10^{4}$ ,while the mean value of weight average molecular weight of *Acacia* Senegal $9\times10^{5}$ (Karamalla *et al* 1998).

An analytical study has been made of gum specimens from *Combretum glutinosum, Combretum fragrans, Combretum collinum* and *Combretum hartmannianum*.( Anderson and Bell 1977). In comparison of *Combretum* species with the genus *Acacia,* both botanical classification and synonymy, and the chemistry, are more complex. 4-O- Methylglucuronic acid, glucuronic acid, galacturonic acid,galactose,arabinose and rhamnose are present in all the specimens studied .In addition, mannose and xylose are present in the gum from *Combretum collinum,* which is therefore similar to *Combretum hartmannianum*. The data from the specimens studied showed that *Combretum* gum exudates are more viscous, of higher molecular weight, and more acidic than those of acacia group.

Table 4.12 shows the comparison with *Combretum* species studied by Anderson, and specimens of gum from *Combretum glutinosum* used in this study, the two specimens were collected from widely differing geographical locations (Nigeria-Sudan), are very similar, although Sudanese sample of *Combretum glutinosum* possesses high Nitrogen % and Protein%**.** Molecular weight in this study number average molecular weight. Molecular weight in pervious published results weight average molecular weight (Anderson, Bell *et al* 1977) and ( Mubasher 2011).

The two specimens of gum from *Combretum fragrans,* from widely differing geographical location, are closely similar, although Sudanese sample of *Combretum fragrans* is of considerably higher Molecular weight . In contrast, the gum from *Combretum collinum* differs extensively from that of its subsp.*hypopilinum*. High values of limiting viscosity number shown by the gums from those species of *Combretum* ,the value for the least viscous gum *Combretum collinum* subsp.*hypopilinum* (60 ml.g $^{-1}$) is much higher than values recorded for *Acacia* gums (4-24 ml.g $^{-1}$) ,these high viscosities are reflected in high Molecular weight; the molecular weight of *Combretum collinum* gum ( **$116 \times 10^5$**) is the highest observed in this analytical data for *Combretum* gums exudates. (Anderson and Bell 1977).

| parameter | *C.glutinosum* | *C.glutinosum* | *( A) C. fragrans* | *(B ) C. fragrans* | *C. collinum* | *C. collinum subsp. hypopilinumm* | *C. hartmannianum* |
|---|---|---|---|---|---|---|---|
| **Origin** | **Sudan-Eldamazine** | **nigeria** | ***Senegal Tanzania*** | **Sudan-Rashad** | ***Senegal Tanzania*** | **-** | **-** |
| **Moisture content%** | **7.96** | **11.9** | **12.5** | **12.0** | **8.9** | **13.8** | **8.3** |
| **Ash%** | **4.51** | **3.3** | **7.7** | **8.3** | **3.3** | **11.7** | **3.7** |
| **Nitrogen %** | **0.37** | **0.07** | **0.17** | **0.18** | **0.13** | **0.10** | **0.61** |
| **Protein%( N×6.25)** | **2.43** | **0.4** | **1.1** | **1.1** | **0.8** | **0.6** | **3.8** |
| **$[\alpha]_D$ in Water, degrees** | **-** | **-9** | **+35** | **+41** | **-81** | **+53** | **-35** |
| **Intrinsic Viscosity, [η] ml/g** | **-** | **75** | **162** | **170** | **312** | **60** | **63** |
| **Molecular Weight $M_w$** | **$1.8\times10^4$** | **$5.3\times10^5$** | **$17\times10^5$** | **$49\times10^5$** | **$116\times10^5$** | **$7.3\times10^5$** | **$6.4\times10^5$** |
| **Acid equivalent weight** | **1524.2** | **1073** | **505** | **487** | **1405** | **398** | **1173** |
| **Uronic acid** | **15.52** | **16.4** | **35.0** | **36.2** | **12.5** | **44.3** | **15** |
| **Sugar composition after hydrolysis (%)** | | | | | | | |
| **4-O- Methylglucuronic acid** | **-** | **3.5** | **5.1** | **6.4** | **2.7** | **8.8** | **1.5** |
| **Glucuronic acid** | **-** | **8.9** | **19.7** | **19.8** | **6.7** | **23.9** | **7.4** |
| **Galacturonic acid** | **-** | **4.0** | **10.2** | **10.0** | **3.1** | **11.6** | **6.1** |
| **Galactose** | **33** | **40** | **34** | **34** | **22** | **36** | **22** |
| **Arabinose** | **56.1** | **31** | **14** | **16** | **47** | **5** | **43** |
| **Rhamnose** | **10.9** | **13** | **15** | **14** | **8** | **13** | **4** |
| **Mannose** | **-** | **-** | **2** | **-** | **9** | **1** | **10** |
| **Xylose** | **-** | **trace** | **trace** | **trace** | **3** | **1** | **6** |

**Table 4.12 Analytical data of *combretum* species**

Optical rotation range from -81° to +53° for *Combretum* gums exudates, the value for *Combretum colli*
the highest negative rotation recorded for a gum exudates (-81°). The uronic acid content also varies ov
from values that occur commonly within the Acacia group (12-16%) to very high values (36.2-44.3%).
a reasonable correlation between the ash content of the purified gums and their uronic acid contents, as i
genus Acacia. Of the species studied, only the gums from *Combretum hartmannianum* and *Combretum*
*Hypopilinumm* contain small quantities of mannose and xylose. (Anderson and Bell 1977)
The family *Combretaceae* (Order, Myrtales), the genus *Combretum* Loefi is the largest and most compl
considerable inter-tree variation, and a wide variation in individual analytical parameters for example ni
specific rotation(+ 53° to-81°), therefore *Combretum glutinosum* differ greatly from gum Arabic ( Acac
of quality, solution properties and values of physical constants.

### 4.3 In *vitro* cytotoxicity

Cytotoxic potential of *Combretum glutinosum* gum, was assessed by MTT reduction assay using two
melanocytes cell line (HFB4), baby hamster normal kidney fibroblast cell line (BHK), in addition t
cells; human colon carcinoma cell line ($HCT_{116}$) and human hepatocellular carcinoma cell line (HEPG
the anticancer efficiencies of the gum samples.
Cell viability was measured by MTT assay and cell viability of non –treated cells was considered as 10
on the selective ability of living cells to reduce only the yellow soluble salt of MTT to apurple-blue
precipitate.The viable cell number is proportional to the production of formazan salts. The advantage
accuracy and reliability and the saving

of time.

### 4.3.1 Cytotoxicity measurements

#### 4.3.1.1 Human normal baby hamster kidney fibroblast cell line (BHK)

Table 4.13 and Figure 4.5; show the effects of *Combretum glutinosum* gum collected from Blue Nile s baby hamster normal kidney fibroblast cell line (BHK). The results show that *Combretum glutinosun* effect on the BHK cell line. The cytotoxicity of the gum is expressed in terms of relative cell viabi comparison to control cells. The percentage of cytotoxicity was calculated considering the control as The results indicated that cytotoxicity represented by median growth inhibitory concentration (I producing 50% cytotoxic.

The half maximal inhibitory concentration ($IC_{50}$) was found to be 41.5 µg $ml^{-1}$ in the category range substance for BHK cell line. The percentages of survived cells in comparison with the control were 2 and 15.9% for concentrations of 62.5, 125, 250 and 500 µg $ml^{-1}$ respectively.

The $IC_{50}$ of the gum collected from Blue Nile state, season 2008 was 175 µg $ml^{-1}$ and the percentages o exposure to different concentrations of gum, in comparison with the control were 79.4%, 56.9%, concentrations of 62.5, 125, 250 and 500 µg $ml^{-1}$ respectively as shown in Table 4.14 and Figure 4.6 this gum is also categorized in the range of potentially toxic substance for BHK cell line. The higher inhibitor, the more agonist activity will be lowered. So the results showed that the order of cytotox season 2007> season 2008.

## GUM CYTOTOXICITY

**Table 4.13 : The relationship between *Combretum glutiosum* gum concentration season 2007 and baby hamster normal kidney fibroblast cell line (BHK) surviving rate.**

| Conc. µg/ml | BHK-1 |
|---|---|
| 0 | 1 |
| 62.5 | 0.286557 |
| 125 | 0.253909 |
| 250 | 0.159154 |
| 500 | 0.159567 |

## BHK

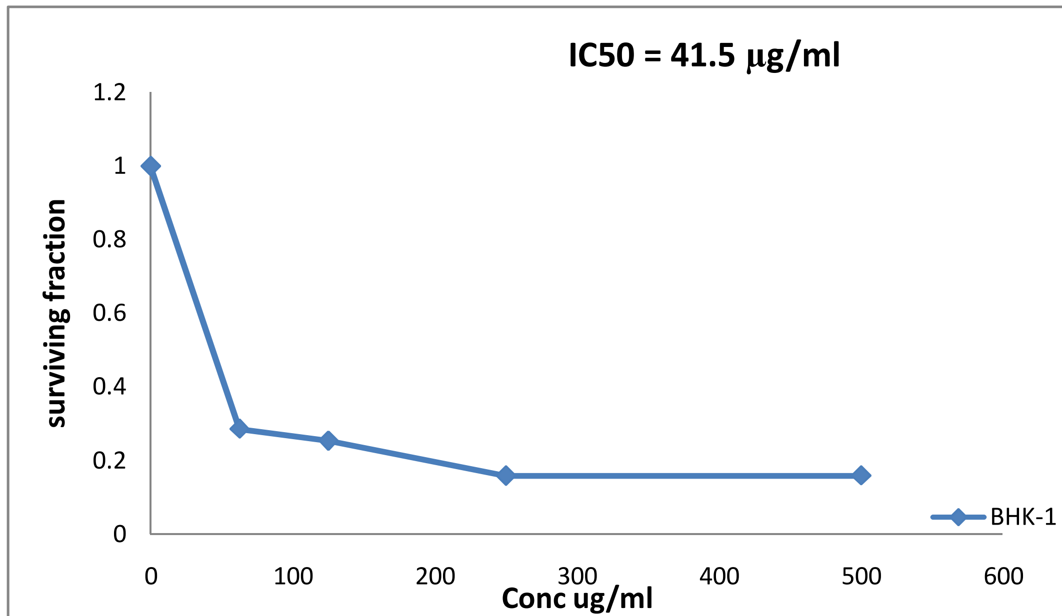



**Figure 4.5 : The relationship between *Combretum glutiosum* gum concentration season 2007 and baby hamster normal kidney fibroblast cell line (BHK) surviving rate.**

## GUM CYTOTOXICITY

**Table 4.14: The relationship between *Combretum glutinosum* gum concentration season 2008 and baby hamster normal kidney**

**fibroblast cell line (BHK) surviving rate.**

| Conc. µg/ml | BHK-4 |
|---|---|
| 0 | 1 |
| 62.5 | 0.799742 |
| 125 | 0.569881 |
| 250 | 0.410005 |
| 500 | 0.159515 |

**BHK**

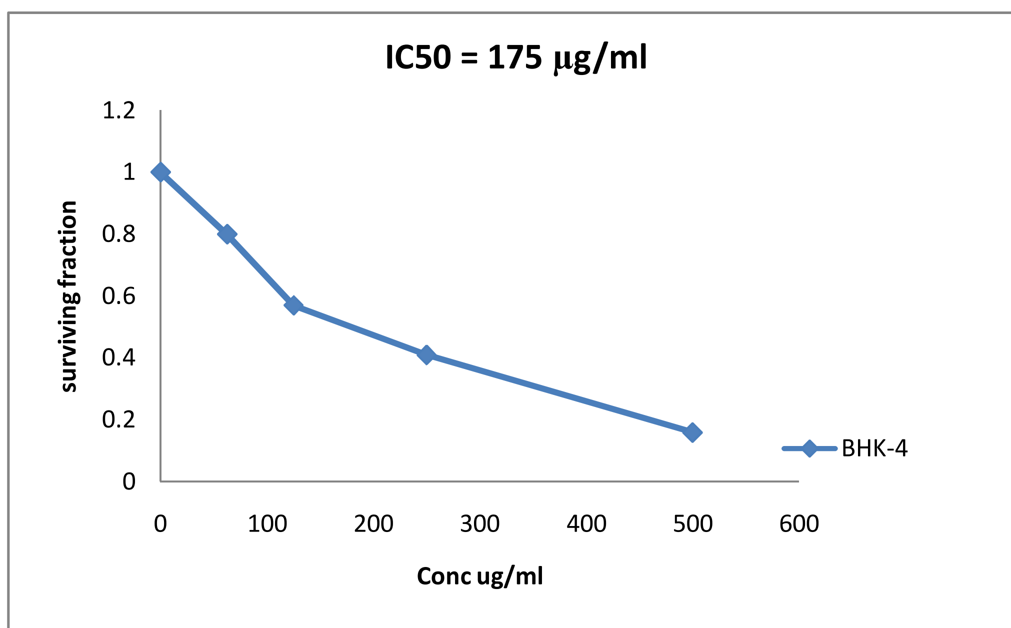


**Figure 4.6 : The relationship between *Combretum glutinosum* gum concentration season 2008 and baby hamster normal kidney fibroblast cell line (BHK) surviving rate.**

### 4.3.1.2 Human normal melanocytes cell line (HFB4)

Table 4.15 and Figure 4.7; show the effects of *Combretum glutinosum* gum collected from Blue Nile s the human normal melanocytes cell line (HFB4). The results show that *Combretum glutinosum* gum ha the HFB4 cell line. The cytotoxicity of the gum is expressed as the same as expressing BHK normal c didn't reach to 50% inhibition of cell growth from control cells. To evaluate and determine the values o *glutinosum* gum on the human normal melanocytes cell line (HFB4) higher concentration than 500 µg n The percentages of survived cells after exposure to different concentrations of gum were 92.3%, 82.2% for concentrations of 62.5, 125, 250 and 500 µg $ml^{-1}$ respectively.

At the same the gum collected from Blue Nile state, season 2008 didn't reach to 50% inhibition of cell cells as shown in Table 4.16 and Figure 4.8. To evaluate and determine the values of $IC_{50}$ of *Combretum* the human normal melanocytes cell line (HFB4) concentration higher than 500 µg $ml^{-1}$ is needed.

The percentages of survived cells after exposure to different concentrations of gum were 92.3%, 72.4%
for concentrations of 62.5, 125, 250 and 500 µg $ml^{-1}$ respectively.

## GUM CYTOTOXICITY

**Table 4.15: The relationship between *Combretum glutinosum* gumconcentration season 2007 and the human normal melanocytescell line (HFB4) surviving rate.**

| Conc. µg/ml | HFB4-1 |
|---|---|
| 0 | 1 |
| 62.5 | 0.923452 |
| 125 | 0.724638 |
| 250 | 0.796245 |

| 500 | 0.740909 |
|---|---|

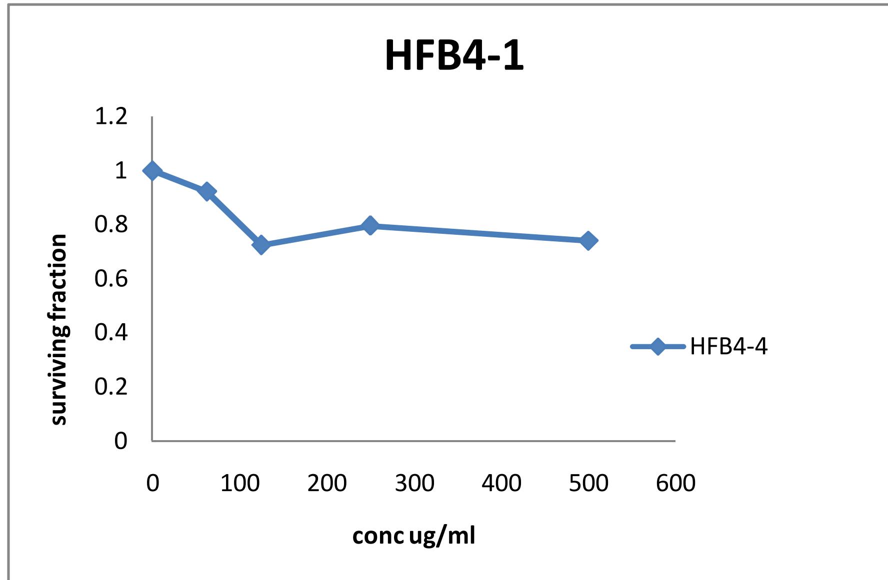



**Figure 4.7 : The relationship between *Combretum glutinosum* gum concentration season 2007 the human normal melanocytes cell line (HFB4) surviving rate.**

# GUM CYTOTOXICITY

**Table 4.16: The relationship between *Combretum glutinosum* gum concentration season 2008 and the human normal melanocytes cell line (HFB4) surviving rate.**

| Conc. µg/ml | HFB4-4 |
|---|---|
| 0 | 1 |
| 62.5 | 0.923452 |
| 125 | 0.724638 |
| 250 | 0.796245 |
| 500 | 0.740909 |

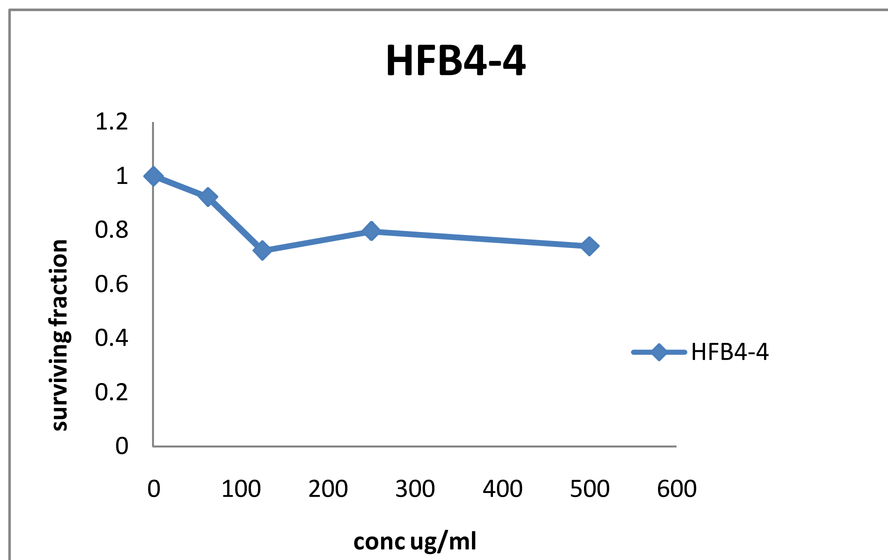


**Figure 4.8: The relationship between *Combretum glutinosum* gum concentration season 2008 the human normal melanocytes cell line (HFB4) surviving rate.**

### 4.3.2 Anticancer effect of gum samples

Two types of human carcinoma cell lines; human hepatocellular carcinoma cell line (HEPG2), human c
line ($HCT_{116}$) treated with different concentration of gum samples, to investigate the effect of gum samp
cancer cell.

#### 4.3.2.1 Human hepatocellular carcinoma cell line (HEPG2)

The cytotoxic effect of *Combretum glutinosum* gum was tested against human hepatocellular carcinom
and human colon carcinoma cell line ($HCT_{116}$). The viable cell number is proportional to the production

Table 4.17 and Figure 4.9; show the effects of *Combretum glutinosum* gum collected from Blue Nile s
the human hepatocellular carcinoma cell line (HEPG2). The results showed that there was gradual dec
of cancer cells with increasing the concentration of the gum; e.g. when the concentration of the gun
viability carcinoma cell lines were inhibited. $IC_{50}$ of the gum was 54 μg ml-1. The percentages of
exposure to different concentrations of gum, in comparison with the control were 41.4%, 35.2%, 28
concentrations of 62.5, 125, 250 and 500 μg $ml^{-1}$ respectively.

The $IC_{50}$ of the gum collected from Blue Nile state, season 2008 was 49.5 μg $ml^{-1}$ and the percentages o
exposure to different concentrations of gum, in comparison with the control were 37 %, 17.7%, 1
concentrations of 62.5, 125, 250 and 500 μg $ml^{-1}$ respectively as shown in Table 4.18 and Figure 4.10.

The cytotoxicity of *Combretum glutinosum* gum for two seasons is categorized in the range of potent
for HEPG2 cell line.

## GUM CYTOTOXICITY

**Table 4.17: The relationship between *Combretum glutinosum* gum concentration season 2007 and the human hepatocellular carcinoma cell line(HEPG2) surviving rate.**

| Conc. µg/ml | HEPG2-1 |
|---|---|
| 0 | 1 |
| 62.5 | 0.414571 |
| 125 | 0.35219 |
| 250 | 0.284762 |
| 500 | 0.243 |

**HEBG2-1**

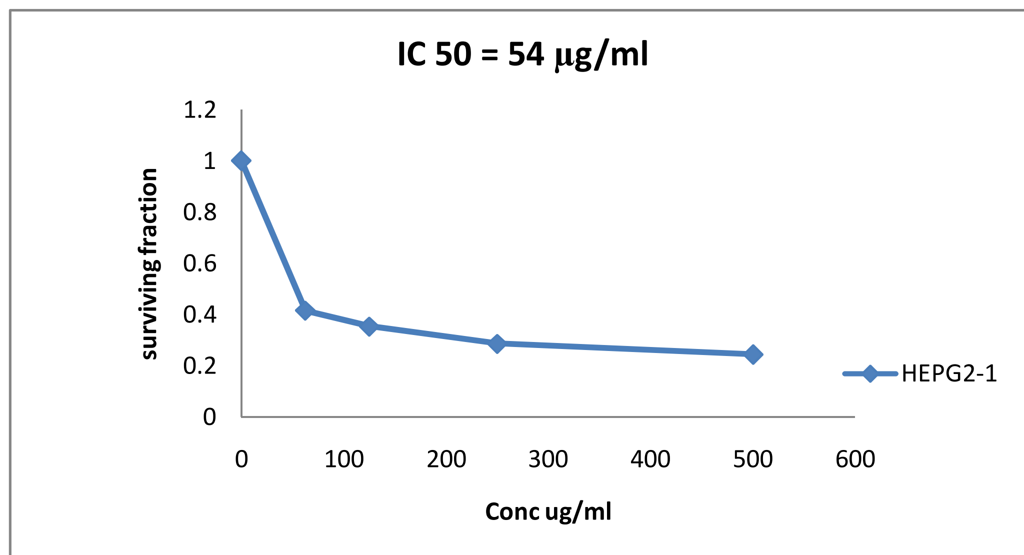


**Figure 4.9 : The relationship between *Combretum glutinosum* gum concentration season 2007 and human hepatocellular carcinoma cell line (HEPG2) surviving rate.**

## GUM CYTOTOXICITY

**Table 4.18: The relationship between *Combretum glutinosum* gum concentration season 2008 and the human hepatocellular carcinoma**

| Conc. μg/ml | HEPG2-4 |
|---|---|
| 0 | 1 |
| 62.5 | 0.370619 |
| 125 | 0.177619 |
| 250 | 0.189667 |
| 500 | 0.280476 |

**HEPG2-4**

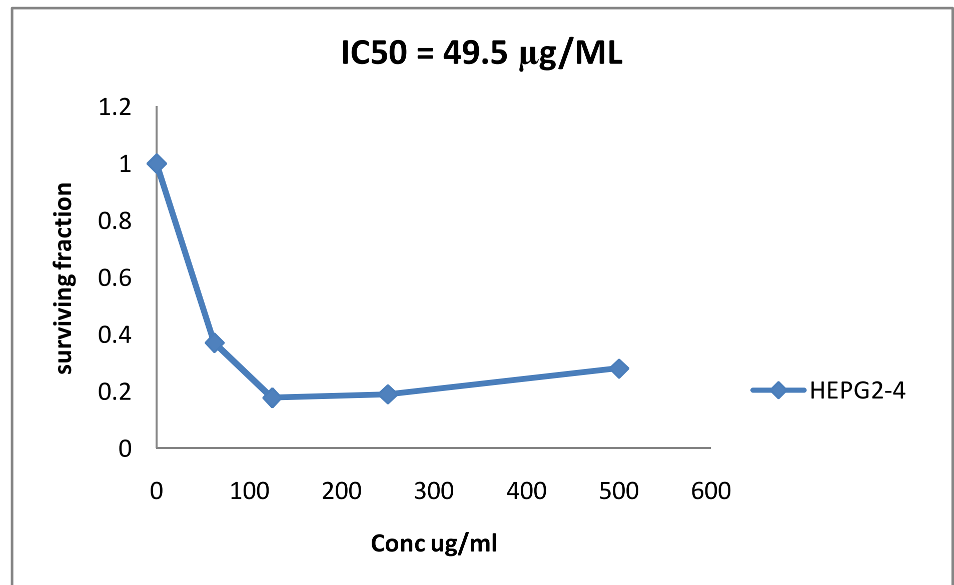


**Figure 4.10 : The relationship between *Combretum glutinosum* gum concentration season 2008 and human hepatocellular carcinoma cell line (HEPG2) surviving rate.**

### 4.3.2.2 Human colon carcinoma cell line ($HCT_{116}$)

In cytotoxic studies, the effect of *Combretum glutinosum* gum solutions on human colon carcinoma cell line ($HCT_{116}$) shown in Table 4.19 and Figure 4.11.

The half maximal inhibitory concentration ($IC_{50}$) was found to be 50 µg $ml^{-1}$ for the gum collected f season 2007 in the category range of toxic substance for $HCT_{116}$ cell line. The percentages of survived with the control were 20.0%, 18%, 17% and 20% for concentrations of 62.5, 125, 250 and 500 µg $ml^{-1}$ r The $IC_{50}$ of *Combretum glutinosum* gum collected from Blue Nile state, season 2008 was found to b $HCT_{116}$ cell line respectively, in the category range of toxic substance for human colon carcinoma cel

percentages of survived cells in comparison with the control were 17%, 13%, 32% and 31% for concent
250 and 500 µg $ml^{-1}$ respectively as shown in Table 4.20 and Figure 4.12.

## GUM CYTOTOXICITY

**Table 4.19: The relationship between *Combretum glutinosum* gum concentration season 2007 and the human colon carcinoma cell line ($HCT_{116}$) surviving rate**

| Conc. μg/ml | HCT-1 |
|---|---|
| 0 | 1 |
| 62.5 | 0.206194 |
| 125 | 0.184905 |
| 250 | 0.17566 |
| 500 | 0.203429 |

**HCT 116**

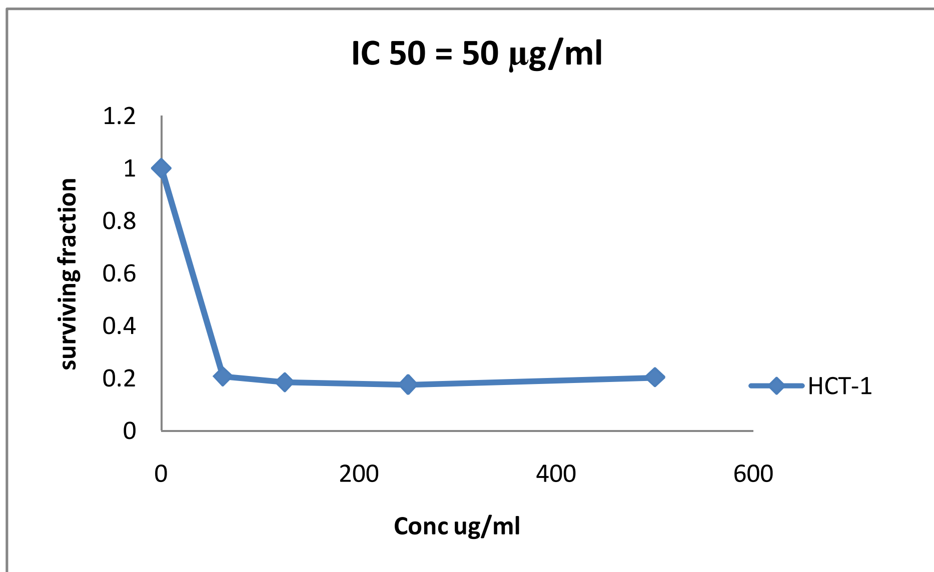


**Figure 4.11 : The relationship between *Combretum glutinosum* gum concentration season 2007 and and the human colon carcinoma cell line ($HCT_{116}$) surviving rate.**

## GUM CYTOTOXICITY

**Table 4.20: The relationship between *Combretum glutinosum* gum concentration season 2008 and the human colon carcinoma cell line ($HCT_{116}$) surviving rate.**

| Conc. µg/ml | HCT-4 |
|---|---|
| 0 | 1 |
| 62.5 | 0.179274 |
| 125 | 0.131367 |
| 250 | 0.328208 |
| 500 | 0.313809 |

## $HCT_{116}$

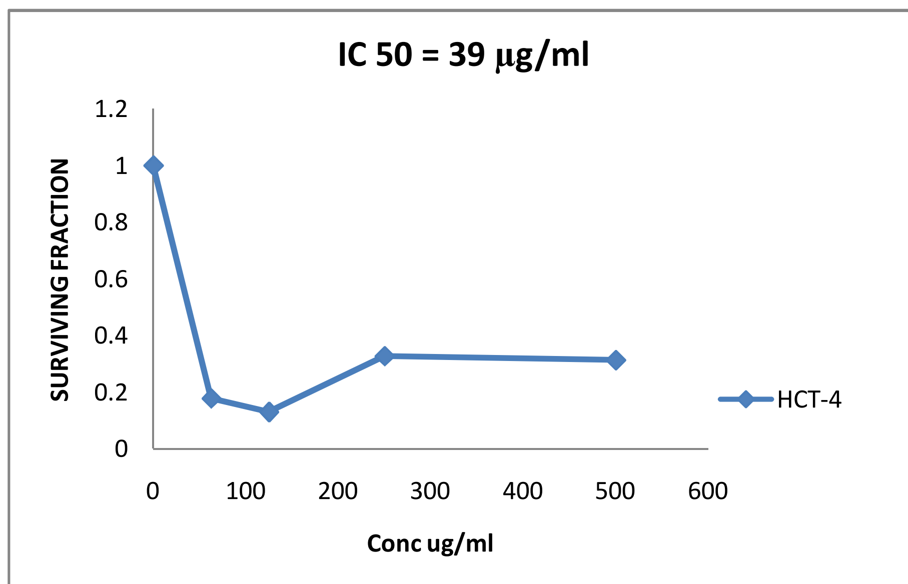



**Figure 4.12 : The relationship between *Combretum glutinosum* gum concentration season 2008 and the human colon carcinoma cell line ($HCT_{116}$) surviving rate.**

### 4.3.3 Cytotoxic results of *Combretum glutinosum* gum and pervious cytotoxic

**results**

Cytotoxic results of *Combretum glutinosum* gum in comparison with pervious cytotoxic results of *Acac*
treated with different doses of gum concentration and the same types of normal and cancer human cell li

**Table 4.21: Results of $IC_{50}$ (µg/ml) *Combretum glutinosum* and $IC_{50}$ (µg/ml) *Acacia senegal*.**

| Cell \ $IC_{50}$ | $IC_{50}$ (µg/ml) *Combretum glutinosum*2007* | $IC_{50}$(µg/ml) *Combretum glutinosum*2008.* | Average ($IC_{50}$) | $IC_{50}$(µg/ml) *Acacia senegal*** |
|---|---|---|---|---|
| HFB4 | - | - | - | 845 |
| BHK | 41.5 | 175 | 108.25 | 629 |
| HEPG2 | 54 | 49.5 | 51.75 | 900 |
| HCT116 | 50 | 39 | 44.5 | 938 |

* doses of *Combretum glutinosum* gum 62.5-500(µg/ml).
** doses of *Acacia senegal* gum 250-1000(µg/ml).

The results showed that the $IC_{50}$ of the *Acacia senegal* and *Combretum glutinosum* gum on the all c
different range, the higher value of $IC_{50}$ of *Combretum glutinosum* gum (108.25 µg/ml)) is much lov
recorded for *Acacia senegal* gums (938-845 µg/ml).These lower concentrations inhibitor of gum are
substance. The cytotoxic effect of *Acacia senegal* gum in category range of potentially harmful substa
The cytotoxic effect of *Combretum glutinosum* gum in category range of potentially toxic substance for
*Combretum glutinosum* gum has popular uses in Sudan and other countries. Although the direct applic
cells is toxic substance, but it is sensitive to the gastro-intestinal environment so that the oral consump

traditional medicine is highly valued in human. Based on these results, further investigations on the *Co*
gum for finding the most cytotoxic agents.

### 4.3.4 Correlation between $LD_{50}$ and $IC_{50}$

MTT method is recommended by ICCVAM as a protocol for basal cytotoxicity for predicting starting systemic toxicity tests $LD_{50}$ .Results of $IC_{50}$ obtained in this study were applied on Halle's RC pred 1998; spielmann et al., 1999).

Table 4.12 show predicted starting doses of $LD_{50}$ for gum samples from $IC_{50}$ values on baby han fibroblast cell line (BHK) and Tables 4.13, 4.14 show predicted starting doses of $LD_{50}$ for gum samp two cancer cell lines; human hepatocellular carcinoma cell line (HEPG2) and human colon carcinoma ce

**Table 4.22:** Prediction of $LD_{50}$ starting doses from $IC_{50}$ values on baby hamster normal kidney fibroblast c Halle's RC prediction model.

| *Combretum* samples | $IC_{50}$ µg/ml | $IC_{50}$ g/l | $LD_{50}$ g/Kg | Prediction of $LD_{50}$ starting doses g/Kg |
|---|---|---|---|---|
| *Combretum glutinosum* season 2007 | 41.5 | 0.0415 | 0.0214 | 0.015 |
| *Combretum glutinosum* season 2008 | 175 | 0.175 | 0.04 | 0.03 |

doses: 62.5, 125, 250 and 500 µg ml.
doses: 0.0625, 0.125, 0.25 and0.5g/Kg
*Estimated* $LD_{50}$ = 0.0214 and 0.04 Starting dose= one default dose below the estimated $LD_{50}$.(0.015-0.03).

**Table 4.23:** Prediction of $LD_{50}$ starting doses from $IC_{50}$ values on human hepatocellular carcinoma cell Halle's RC prediction model.

| *Combretum* samples | $IC_{50}$ µg/ml | $IC_{50}$ g/l | $LD_{50}$ g/Kg | Prediction of $LD_{50}$ |
|---|---|---|---|---|

| | | | | starting doses g/Kg |
|---|---|---|---|---|
| *Combretum glutinosum* season 2007 | 54 | 0.054 | 0.024 | 0.015 |
| *Combretum glutinosum* season 2008 | 49.5 | 0.0495 | 0.023 | 0.015 |

Default doses: 62.5, 125, 250 and 500 µg ml.
Default doses: 0.0625, 0.125, 0.25 and0.5g/Kg
*Estimated* $LD_{50}$ = 0.024 and 0.023 Starting dose= one default dose below the estimated $LD_{50}$. (0.015).

**Table 4.24:** Prediction of $LD_{50}$ starting doses from $IC_{50}$ values on human colon carcinoma cell line (HCT
RC prediction model.

| *Combretum* samples | $IC_{50}$ µg/ml | $IC_{50}$ g/l | $LD_{50}$ g/Kg | Prediction of $LD_{50}$ starting doses g/Kg |
|---|---|---|---|---|
| *Combretum glutinosum* season 2007 | 50 | 0.05 | 0.023 | 0.015 |
| *Combretum glutinosum* season 2008 | 39 | 0.039 | 0.021 | 0.015 |

Default doses: 62.5, 125, 250 and 500 µg ml.
Default doses: 0.0625, 0.125, 0.25 and0.5g/Kg
*Estimated* $LD_{50}$ = 0.023 and 0.021 Starting dose= one default dose below the estimated $LD_{50}$.(0.015).

## 4.4 Conclusion and Suggestions for Further Work

### 4.4.1 Conclusion

- Physicochemical results showed no significant differences within samples collected into two seas

- Tannin, starch and dextrin were not detected in all gum samples.
- *Combretum glutinosum* gum dissolved perfectly in bases, and shows low solubility in water.
- *Combretum glutinosum* gum is a salt of calcium, magnesium, and potassium.
- Heavy metals such as Cr, Cd, and Co were found in traces in *Combretum glutinosum* gum.
- FT-IR spectrum for two seasons showed similar functional groups results.
- All gum samples showed $IC_{50}$ less than 100 µg/ml, except one.
- All gum samples showed low $LD_{50}$ in range 0.02-0.03 g/Kg.
- *In vitro* method could be used for predication of $LD_{50}$ Starting dose of other gum species, consid
  and low cost of *In vitro*
  compare *to in vivo* method.

**4.4.2 Suggestions for Future Work**.

- *In vivo* cytotoxicity tests are required to complete other factors influencing toxicity of *Combr*
  gum.
- More extensive studies are needed to examine chronic, subchronic and cytotoxicity of *Combr*
  gum .