INVESTIGATING THE ANTICARCINOGENIC ROLE OF *SALIX AEGYPTIACA* L. IN COLORECTAL CARCINOMA

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ABSTRACT

INVESTIGATING THE ANTICARCINOGENIC ROLE OF *SALIX AEGYPTIACA* L. IN COLORECTAL CARCINOMA

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In this study, extracts from bark, leaves and catkins of *Salix aegyptiaca* L. were investigated for their antioxidant content by 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) free radical quenching assay, total phenolic and total flavonoid assays. The highest antioxidant activity (19 μg/ml IC_{50} for inhibition of DPPH radical activity), total phenolic content (212 mg gallic acid equivalents/g of dried extract) and total flavonoid (479 mg catechin equivalents/g of dried extract) was observed in the ethanolic extract of bark.

High performance liquid chromatography (HPLC) analyses revealed the presence of gallic acid, caffeic acid, vanillin and p-coumaric acid, myricetin, catechin, epigallocatechin gallate, rutin, quercetin as well as salicin.

In addition, the anti-proliferative effects of the ethanolic extracts on colorectal cancer cell lines (HCT-116 and HT-29) were examined by an MTT cell viability assay while their apoptotic effects were assayed by acridine orange staining and

caspase 3 activity. The results indicate that the ethanolic extract of bark of *S. aegyptiaca* can strongly inhibit cell proliferation and induces apoptosis in a dose dependent manner on both cell lines.

We propose that extracts from this plant may be utilized as a source of health promoting antioxidants. Our data provide a perspective for more detailed study of biochemical pathways associated with the cancer preventive effects of active components of the extracts from *S. aegyptiaca*.

Key words: Salix aegyptiaca, Antioxidants,Phenols, flavonoids, Growth Inhibition, Apoptosis, Colon Cancer

ÖZ

SALİX AEGYPTİACA L. İN KOLOREKTAL KANSER ÜZERİNE ANTİKARSİNOJENİK ROLUNUN ARAŞTIRILMASI

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Bu çalışmada *Salix aegyptiaca* L bitkisinin kabuk, yaprak ve çiçek ekstraktlarının antioksidan içerikleri 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) serbest radikal sönümlenme, total flovanoid ve total fenolik içerik deneyleri ile araştırılmıştır. En yüksek antioksidan aktivite (10 μg/ml IK 50 DPPH radikal aktivite inhibisyonu), total fenolik içerik (121 mg/gallik asit eşleniği / gram kurutulmuş ekstrakt) ve toplam flovanoid içeriği (479 mg katekin eşleniği /gram kurutulmuş ekstrakt) kabuk etanolik ekstraktlarında gözlemlenmiştir. Yüksek Performans Sıvı Kromatografisi çalışmaları da etanolik kabuk akstraktlarında salisin gallik asit, kafeik asit, vanilin, p-kumarik asit, mirisetin, katekin, epigallokatekin gallat, kuersetin varlığını göstermiştir.

Buna ek olarak, etanolik ekstraktların kolon kasneri hücre hatlarındaki (HCT-116 ve HT-29) anti-proliferatif özelliklerine MTT hücre proliferasyonu deneyleri ile bakılmış diğer taraftan apoptotik özellikleri ise akridin oranj boyaması ve kaspaz 3 aktivitesi ölçümü ile araştırılmıştır.

Sonuçlar göstermiştir ki: *S. Aegyptiaca* bitkisinden hazırlanan etanolik ekstraktlar hücre çoğalmasını önemli ölçüde inhibe etmiş ve apoptozu doza ve zamana bağlı olarak indüklemiştir. Biz bu bitkinin etanolik ekstraktlarının sağlıklı antioksidan

olarak kullanılabileceğini düşünmekteyiz. Elde ettiğimiz veriler *S. Aegyptiaca* ekstraktlarındaki kanser önleyici aktif bileşenler ve ilgili biyokimyasal yolaklar ile daha detaylı bir çalışma yapmak için yeni bir bakış açısı sağlamıştır.

Keywords: Salix aegyptiaca L antioksidan, fenolik bileşikler, Böyüme İnhibisiyon, Apoptoz, Kolon Kanseri

DEDICATED

TO

MY FAMILY

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CHAPTER 1

INTRODUCTION

'*Let food be your medicine and medicine your food*'.

Hippocrates, 4th century BC.

In this study, we have characterized extracts from bark, leaves and catkins of *Salix aegyptiaca* L for their antioxidant, phenolic and flavonoid contents and their ability to exert anti-proliferative and pro-apoptotic effects on colorectal cancer cell lines.

This chapter summarizes the available literature on free radicals, antioxidants, and phenolic and flavonoid compounds including salicin and its derivatives and their effects on diseases. In addition, the possible links between antioxidant compounds and their anti-proliferative effects is also discussed.

1.1 FREE RADICALS AND ANTIOXIDANT DEFENSE

Free radicals are defined as molecules or molecular fragments containing one or more unpaired electrons in their atomic or molecular orbitals (Halliwell, 1999). The unpaired electrons make free radicals highly reactive and unstable. Oxygen-derived free radicals, also called reactive oxygen species (ROS), are considered as the most important and varied class of radicals, present in biological systems (Miller, Buettner & Aust, 1990).

Molecular oxygen metabolisms can lead to the generation of several different reactive oxygen species (ROS) (Halliwell, 1999) such as superoxide anion radical ($O_2^{\cdot}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($^{\cdot}OH$).

Normally, during aerobic metabolism the production of ROS is tightly controlled, although a basal amount is constantly produced in the body (Weinberg, 1996). The presence of these intracellular active oxygen species may result in a wide range of modifications in structure and function of macromolecules and alterations in biochemical pathways related to cell signaling, cell growth, proliferation, and apoptosis. Consequently, organisms have developed a variety of cellular antioxidant defense systems in order to detoxify and eliminate these reactive species. Any change in the balance between antioxidant defenses and radical activity of ROS may result in a condition referred to as oxidative stress.

The harmful effects of ROS are usually a result of dysregulated generation. However, when their production is tightly controlled, they can also be implicated as signaling molecules in several intracellular signaling pathways (Klein & Ackerman, 2003; Nohl, Kozlov, Gille & Staniek, 2003).

1.2 Endogenous sources of ROS:

ROS are produced in living cells from endogenous and exogenous sources. Endogenously produced ROS include hydrogen peroxide (H_2O_2**)** and superoxide anion ($O_2^{\cdot -}$), which are generated as products of cellular metabolism. Some of these pathways have been described below:

1.2.1 Mitochondrial electron transport chain:

During energy transduction in the mitochondrion, a small number of electrons "leak" prematurely, resulting in the formation of oxygen free radical superoxide, which has been implicated in the pathophysiology of a variety of diseases (Kovacic, Pozos, Somanathan, Shangari & O'Brien, 2005; Valko, Izakovic, Mazur, Rhodes & Telser, 2004). As much as 1–3% of all electrons in the transport chain "leak" to generate superoxide instead of contributing to the reduction of oxygen to water. Superoxide is produced from both complex I (NADH: ubiquinone oxidoreductase), and complex III (coenzyme Q: cytochrome c— oxidoreductase) of the electron transport chain by leakage of electron to oxygen. Once in its anionic form, oxygen has a strong charge,

which enables it to readily cross the inner mitochondrial membrane (Muller, Liu & Van Remmen, 2004). Alternatively, hydrogen peroxide may be converted into water by the enzyme catalase or glutathione peroxidase.

1.2.2 Inflammation:

A significant source of free radicals in the body is the phagocytic cells (ie macrophages and dendritic cells). A phenomenon called "respiratory (oxidative) burst" in this type of cell results in the induction of short-lived free radicals, which are used as defense mechanisms against microbial pathogens since they are highly toxic to bacteria, fungi and some protozoa. In addition, the combination of nitric oxide, which is produced by neutrophils in large quantities, with the products of the oxygen reduction system, leads to the formation of harmful peroxynitrites, which are also effective defense molecules (Valko, Leibfritz, Moncol, Cronin, Mazur & Telser, 2007).

1.2.3 Peroxisomes:

Peroxisomes are membrane-bound organelles found in eukaryotic cells. These organelles are capable of both generating and scavenging ROS via the production and decomposition of H_2O_2 during oxygen consumption and prevention of it's accumulation due to the functions of the enzyme catalase (Valko et al., 2004; Wanders & Waterham, 2006). The balance between the processes of ROS generation and consumption by peroxisomes is crucial for the appropriate function of the cells and its dysfunction has been implicated in aging and carcinogenesis (Wanders & Tager, 1998).

1.2.4 Intermediate Metal Ions:

In vivo, the increased level of superoxide radicals in stress conditions results in the release of iron ions from iron-containing molecules. In the Fenton reaction (reaction 1 below) ferrous iron reacts with hydrogen peroxide and results in the formation of the highly reactive hydroxyl radical (Leonard, Harris & Shi, 2004; Valko, Morris &

Cronin, 2005). The Haber-Weiss reaction results from the reaction between H_2O_2 (hydrogen peroxide) and superoxide ($\bullet O_2^-$) resulting in the formation of $^{\bullet}OH$ (hydroxyl radicals) as shown in reaction 2 (Liochev & Fridovich, 2002).
In addition, in the Haber–Weiss reaction, Fe^{3+} is reduced to Fe^{2+} by superoxide ion (reaction 3).

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$
(2) $O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$
(3) $Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$

1.3 Exogenous Sources of ROS:

Cigarette smoke, air pollutants, U.V. radiation, singlet oxygen (O^-), oxides of nitrogen (NO and NO_2), ionizing radiation, drugs and even alcohol are exogenous sources of ROS. They are characterized by a high level of cytotoxicity and carcinogenic potential, which are the result of their catabolism within the body leading to the generation of large amounts of free radicals (Davies & Pryor, 2005).

1.4 Reactive Oxygen Species Relevant to Human Health

1.4.1 Superoxide Radicals:

The unique electronic configuration of molecular oxygen makes it structurally a radical. Superoxide anion is formed when one electron is added to dioxygen (O_2). This addition of an electron can occur via the activation of oxygen by physical irradiation or during metabolic processes such as mitochondrial respiration and energy transduction, during which leakage of electrons occur, resulting in the conversion of molecular oxygen to superoxide radicals. These reactions produce a primary ROS (Miller et al., 1990) (see Fig. 1). Further this primary reactive molecule can directly or indirectly, through enzymatic processes, interact with other molecules to induce “secondary” ROS (Valko et al., 2005). Superoxide radicals participate in promotion and progression of a variety of disorders. However, they also play a key role in the phagocytic process in the defense against microbial pathogens (Kovacic et al., 2005; Valko et al., 2004).

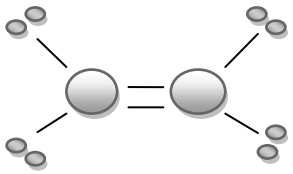
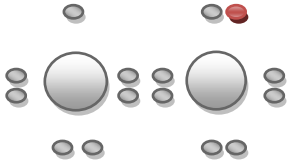
(A)  (B) 

Figure 1. A: Oxygen molecule, B: Superoxide anion

1.4.2 Hydroxyl Radical:

The hydroxyl radical, •OH is a highly reactive and dangerous radical with a very short *in vivo* half-life of approx 10^{-9} s (Pastor, Weinstein, Jamison & Brenowitz, 2000 & Brenowitz, 2000). Due to this very short half-life, its site of reaction is usually very close to its site of formation. The formation of hydrogen peroxide from superoxide anion through the catalyzing function of superoxide dismutase is the first step of hydroxyl radical formation (reaction 1 below). The hydrogen peroxide is catalyzed to water and oxygen by the enzyme catalase (reaction 2). In addition, in a Fenton type reaction, the hydrogen peroxide also readily reacts with ferrous iron (Fe^{2+}) or other transition metal ions to generate hydroxyl radical (reaction 3). Then, the ferric iron (Fe^{3+}) generated in reaction 3 can react with another superoxide anion to form ferrous iron (Fe2+) and molecular oxygen (reaction 4). This new ferrous iron is now ready to interact with another peroxide molecule. Therefore, even small amounts of iron ion can be sufficient to catalyze the formation of huge amount of hydroxyl radicals (Leonard et al., 2004; Liochev & Fridovich, 2002; Valko et al., 2005).

1) $2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$

2) $2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$

3) $H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$

4) $Fe^{3+} + O_2^{\bullet-} \longrightarrow O_2 + Fe^{2+}$

1.4.3 Reactive Nitrogen Species (RNS)

$NO^{\bullet}$ with an unpaired electron is a small molecule generated in biological tissues as a result of the metabolism of arginine to citrulline in a reaction catalyzed by specific

nitric oxide synthases (NOSs) (Ghafourifar & Cadenas, 2005). Nitric oxide radicals are abundantly present as important oxidative signaling molecules in the biological system and play a key role in a wide range of physiological processes, including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation (Bergendi, Benes, Durackova & Ferencik, 1999).

1.5 The Functions of Free Radicals:

While at tightly regulated concentrations ROS behave as signaling molecules, excessive production of ROS results in cellular and molecular damage in the body (Valko, Rhodes, Moncol, Izakovic & Mazur, 2006). The accumulation of oxidative damage influences a range of biological processes such as signal transduction and gene expression, mitogenesis, transformation, mutagenesis and cell death (Hunt et al., 1998; Mills et al., 1998).

1.5.1 Oxidative Damage to DNA:

Oxidative DNA damage caused by free radicals is the most frequent damage occurring in aerobic cells. Free radicals are able to induce multiple modifications in the DNA structure resulting in the formation of mutations, deletions, gene amplification and rearrangements.

The accumulation of such damages may eventually lead to the activation of apoptosis signaling pathways and end up with cell death or the enhanced expression of several proto-oncogenes and/or reduced expression of some tumor suppressor genes resulting in carcinogenesis. Therefore, the use of antioxidants and preserving the balance of redox state in biochemical pathways in the cell are critical factors in therapeutic approaches (Mates & Sanchez-Jimenez, 2000).

1.5.2 Oxidative Damage to Lipids:

Lipids are the other cellular components, which are influenced by the attack of ROS, especially metal-induced ROS. Polyunsaturated fatty acids (PUFA) produced from membrane phospholipids by the action of phospholipases are highly sensitive to oxidation (Siems, Grune & Esterbauer, 1995). Through a cyclization reaction the

peroxyl radicals are rearranged to endoperoxides (precursors of malondialdehyde) which are then subjected to a peroxidation process leading to the production of malondialdehyde (MDA) end products. Studies indicate that MDA is a mutagenic factor in bacteria and mammalian cells and is carcinogenic in rats (Fedtke, Boucheron, Walker & Swenberg, 1990; Fink, Reddy & Marnett, 1997; Mao, Schnetz-Boutaud, Weisenseel, Marnett & Stone, 1999; Wang, Dhingra, Hittelman, Liehr, deAndrade & Li, 1996).

1.5.3 Oxidative Damage to Proteins:

The side chains of all amino acid residues of proteins, especially cysteine and methionine are extremely vulnerable to oxidation by ROS/RNS (Stadtman, 2004). For instance oxidation of cysteine residues can cause the reversible formation of disulphide complexes between protein thiol groups (–SH) and low molecular weight thiols, such as GSH (*S*-glutathiolation). These disulphide bonds are critical in the tertiary and quaternary structure of proteins. Since protein folding is critical for its function, oxidative damage can cause irreversible damage to proteins (Dalle-Donne, Giustarini, Colombo, Rossi & Milzani, 2003; Dalle-Donne et al., 2005).

1.5.4 ROS and Signal Transduction Pathways:

When stimulated by a wide range of stimuli including cytokines, growth factors and hormones, most cell types elicit a small oxidative burst leading to the generation of a low concentration of ROS (Thannickal & Fanburg, 2000). It is hypothesized that besides the predominant involvement of ROS in inducing cellular damage, the ROS generated via endogenous and constitutive reactions in cells are functional in cell signaling pathways contributing to cell growth and differentiation. ROS can thus play a very important physiological role as secondary messengers (Lowenstein, Dinerman & Snyder, 1994; Storz, 2005).

In contrast to the above-mentioned detrimental effects, the beneficial effects of ROS/RNS (e.g. superoxide radical and nitric oxide) occur at low/moderate concentrations and involve physiological roles in the cellular responses to noxia,

defense against infectious agents, cellular signaling pathways, and the induction of a mitogenic response. One signaling pathway that is commonly activated by ROS is the mitogen activated protein kinase (MAP kinase) pathway (Valko et al., 2007).

When growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF) (Neufeld, Cohen, Gengrinovitch & Poltorak, 1999) or cytokines such as TNF-α, IFN-γ and IL-1 bind to their respective receptors in non phagocytic cells, it results in the generation of ROS. These ROS then behave as secondary messengers resulting in the activation of the MAP-kinase pathway resulting in the activation of several responses such as growth, differentiation, inflammation and apoptosis (Sundaresan et al., 1996).

1.6 ROS and Human Disease

Oxidative stress by disrupting several aspects of cellular functions, play an important role in the induction and progression of various pathological disorders such as cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion injuries, as well as ageing (Dalle-Donne, Rossi, Colombo, Giustarini & Milzani, 2006; Dhalla, Temsah & Netticadan, 2000; Jenner, 2003; Sayre, Smith & Perry, 2001).

1.6.1 ROS and Cancer

A large number of studies emphasize on the detrimental role of ROS on the initiation and progression of cancer via oxidative damage to proteins, particularly transcription factors, which are involved in growth and cell cycle regulation. Therefore, the effects of ROS and oxidative stress on some important transcription factors participating in carcinogenesis are summarized here.

A. AP-1

activator protein 1 (AP-1) is a protein that belongs to the Jun, and Fos subfamilies that is capable of binding to tumor-promoting agent (TPA) or cyclic AMP (cAMP) response elements (Rao, Luo & Hogan, 1997). The transcriptional activity of AP-1 is

increased in the presence of H_2O_2, different cytokines, physical and chemical stresses and in response to some metals (Valko et al., 2006).

The increased activity of AP-1 results in an increase in cellular proliferation. In addition, AP-1 possesses a regulatory effect on apoptosis. It can either induce or inhibit apoptosis depending on the type of stimulus, the duration of stimulation and the pro or anti-apoptotic target genes (Valko et al., 2006).

B. NF-κB:

NF-κB (Nuclear factor kappa B) is a ubiquitous transcription factor, canonically comprising of a heterodimer of p50 and p65 (also called RelA) subunits. This transcription factor is capable of binding to the promoter region of many genes involved in an inflammatory response (Pande & Ramos, 2005). In its inactive form, NF-κB is present in the cytoplasm bound to a protein called inhibitor of κB (IκB). However, in the presence of stimulatory agents such as free radicals, carcinogens and tumor promoters, and the inhibitory effect of IκB is attenuated and the newly released p50 and p65 subunits translocate to the nucleus. There they cause the expression of genes which suppress apoptosis, induce cellular transformation, proliferation, invasion, metastasis, chemoresistance, radioresistance, and inflammation. Reactive oxygen species act as second messengers in participation with tumor necrosis factor alpha (TNF-α) and the cytokine interleukin-1β, to induce NF-κB activation (Baud & Karin, 2001).

Elevated activation of NF-κB has been reported in many types of cancer including; colon, breast, and pancreas. Antioxidants, including l-cysteine, N-acetyl cysteine (NAC), thiols, green tea polyphenols, and vitamin E can block the activation of NF-κB by nearly all stimuli (Storz, 2005; Valko et al., 2006).

C. p53

P53 is a tumor suppressor gene, which has a protective role in cells against tumorigenesis. It induces its effect by arresting the cell cycle when there is DNA damage and maintaining the arrested state until repair occurs (Hofseth, Hussain & Harris, 2004) (Hollstein, Sidransky, Vogelstein & Harris, 1991). According to (Sablina, Budanov, Ilyinskaya, Agapova, Kravchenko & Chumakov, 2005) there is a direct link between the levels of p53 and ROS production. Under stress conditions, when the levels of p53 are high, the protein transcribes apoptotic genes and genes contributing in ROS formation. On the other hand, at low concentrations i.e. low stress situations, it promotes the activity of antioxidant genes.

Taking into consideration the myriads of detrimental effects of free radicals in biological systems, living organisms have developed a variety of antioxidant defense mechanisms against these highly reactive compounds. In addition in recent years, owing to a dramatic increase in the levels of industrial and environmental pollutants and the resultant oxidative damage in cells (Singh et al., 2007), attention has focused on natural sources of antioxidants, specifically plant based antioxidants.

1.7 ANTIOXIDANTS

1.7.1 Natural Antioxidants:

Antioxidants are a wide range of compounds, which are able to destroy and scavenge free radicals in the body, and thereby protect the cells against oxidative damage. Natural antioxidants (enzymatic and non-enzymatic) are found from a variety of endogenous and exogenous sources as described below.

1.7.1.1 Enzymatic Antioxidant Defence Systems:

The most important and well studied enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT).

A. Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) catalyzes the reduction of superoxide anions to hydrogen peroxide and thereby possesses a protective role in defense mechanisms against the toxic effects of oxygen radicals. Furthermore, SOD prevents the formation of hazardous peroxynitrite induced by the reaction between nitric oxide and superoxide anion. Copper-Zinc SOD is the common form of SOD in eukaryotes (Cu-Zn-SOD). The complex of SOD with manganese, iron, or nickel is also present in different cells (Corpas et al., 2006).

B. Catalase

Catalase is a major antioxidant enzyme, which works coupled with SOD. SOD reduces superoxide anion to hydrogen peroxide and catalase induces the decomposition of hydrogen peroxide to water and oxygen (Fig 2). One molecule of catalase is capable of converting millions of molecules of hydrogen peroxide to water and oxygen per second (Valko, 2007).

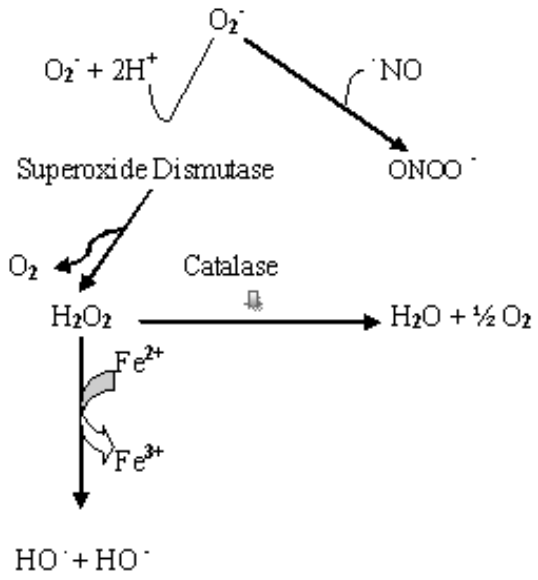


Figure 2: Superoxide dismutase and Nitrogen monoxide compete for superoxide

C. Glutathione (GSH):

Glutathione is a major soluble antioxidant, present in large quantities in the cytosol, nuclei and mitochondria and acts as a cofactor of several detoxifying enzymes (such as glutathione peroxidase) against oxidative stress. It can also be found in the oxidized form of glutathione disulphide (GSSG) (Shen, Dalton, Nebert & Shertzer, 2005). GSH possesses a critical role in the maintenance of redox state of sulphydryl proteins essential for DNA repair and expression (Jones, Carlson, Mody, Cai, Lynn & Sternberg, 2000; Nogueira, Zeni & Rocha, 2004). In addition, because of its antioxidant capacity, it scavenges a wide range of ROS including hydroxyl radical, singlet oxygen and detoxifies hydrogen peroxide and lipid peroxides. Moreover, the reduced GSH is able to reactivate the oxidized form of important antioxidants (e.g. Vitamins C and E) resulting in the production of oxidized GSSG. An enzyme called glutathione reductase regenerates the GSH. However, high concentration of oxidized glutathione may oxidize and damage other enzymes, and the ratio of GSH/GSSG is an indicator of oxidative stress in an organism (Masella, Di Benedetto, Vari, Filesi & Giovannini, 2005; Pastore, Federici, Bertini & Piemonte, 2003).

1.7.1.2 Non-Enzymatic Antioxidants

Non-enzymatic antioxidants can be divided into different categories including:

a) chelating agents which combine with metal ions (e.g. transferrin, albumin, seroplasmin)
b) Vitamins (e.g. Vitamin C, E , A)
c) Vitamin cofactors and minerals (e.g. Rubidium, Vanadium, Zinc, Iron, Selenium and Coenzyme Q10) (Kwun, Park, Jang, Beattie & Kwon, 2005; Valko et al., 2007)
d) Carotenoid terpenoids (e.g. Lycopene, lutein, alfa and beta carotene)
e) Flavonoid polyphenolics (e.g. flavones, flavonols, flavonones, flavonols and their polymers) (Le Marchand, 2002; Valko et al., 2007)
f) Phenolic acids and their esters (e.g. gallic acid, caffeic acid, coumaric acids) (Croft, 1998)

g) Other non-flavonoid phenolics (e.g. curcumin eugenol) (Motterlini, Foresti, Bassi & Green, 2000)
h) Other organic antioxidants (e.g. uric acid, bilirubin, alfa lipoic acid (ALA)) (Packer, Witt & Tritschler, 1995; Stocker, Yamamoto, McDonagh, Glazer & Ames, 1987)

1.7.2 Plant Based Antioxidants:

Epidemiological studies on plant-based diets emphasize the protective role of diets rich in vegetables and fruits, against several disorders, especially cancer. Increasing evidence on this protective role emphasize the necessity to utilize the benefits of these natural compounds in order to control and treat diseases. Bioactive secondary metabolites of these plants generally called phytochemicals, elicit their effects by the suppression of inflammatory processes such as cyclooxygenase-2 expression, detoxification and elevated excretion of carcinogens, inhibition of mitosis and the induction of apoptosis at various stages in the progression and promotion of cancer (Johnson, 2007). Flavonoids are generally considered as strong antioxidants and important elements in the prevention and treatment of a variety of disorders such as cancer. Evidence on the protective effects of flavonoids on cancer will be explained here.

1.7.2.1 Flavonoids

Flavonoids are a large group of plant phenolics with over 5000 members which can be found in most fruits, vegetables and beverages (tea, coffee, beer, wine and fruit drinks) (Hertog, Feskens, Hollman, Katan & Kromhout, 1994; Le Marchand, 2002). Based on their chemical structure, they have been categorized into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. The intake of flavonoids via the diet is said to be higher than that of other antioxidants like vitamin C and E. Moreover, since the intake of flavonoids is independent from the intake of micronutrients such as vitamins A and C, it provides a specificity in epidemiological studies to determine the association of flavonoids and diseases independent of these micronutrients (Le Marchand, 2002).

1.7.2.2 Cancer Protective Effects of Flavonoids

Although there is a wide range of evidence on the protective effects of plant-derived foods in cancer, the current knowledge about the association of specific compounds with these protective effects it is not clear and sufficient. In recent years, growing evidence on the anti-carcinogenic effects of flavonoids has aroused considerable interest to determine the possible mechanisms and biological pathways that are implicated in the effects of these bioactive products (Le Marchand, 2002).

1.7.2.3 Effects of Flavonoids on Cancer-related Pathways

Current studies have shown the interaction of flavonoids with several biological pathways related to cancer including; cell signaling pathways, cell cycle regulation, angiogenesis, oxidative stress, and inflammation. Different flavonoids may insert their beneficial and protective role by interacting with a variety of mechanisms involved in carcinogenesis as detailed below (Omenn et al., 1996; Yang, Landau, Huang & Newmark, 2001).

A. Antioxidant Functions of Flavonoids

Flavonoids act as strong scavengers of most types of oxidizing molecules (Bravo, 1998) implicated in DNA damage and tumorigenesis (Cerutti, 1985). Most chemical carcinogens require transformation into more active forms to be able to bind to and damage DNA. Flavonoids prevent this transformation by activation of detoxifying enzymes as well as inhibition of radical generating enzymes.

The antioxidant capacity of flavonoids is determined by their molecular structure, especially the position of hydroxyl groups, which are critical for their radical scavenging activity (Hertog et al., 1994). According to some scientific reports, the antioxidant capacity of flavonoids can reduce lipid peroxidation by their ability to scavenge hydroxyl and superoxide anions. Flavonoids can act as electron donors and react with free radicals to stabilize them thereby terminate free radical chain reactions (Gonzalez-Segovia, Quintanar, Salinas, Ceballos-Salazar, Aviles-Jimenez & Torres-Lopez, 2008; Robak & Gryglewski, 1988).

B. Inhibition of Cytochrome P450 enzymes

Cytochrome P450 enzymes are a group of bioactivating enzymes that possess a critical role in the activation of a number of carcinogens. Flavonoids such as flavonols, quercetin, kaempferol and galangin, and the flavone apigenin are able to inhibit cytochrome P450 enzymes (Ciolino, Daschner & Yeh, 1999; Kang, Tsai & Lee, 1999; Lautraite, Musonda, Doehmer, Edwards & Chipman, 2002; Muto, Fujita, Yamazaki & Kamataki, 2001).

C. Detoxification and Induction of Antioxidant Enzyme Activity

According to the data from animal and *in vitro* studies, tea flavonoids (catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG)) induce an enhanced activity of several detoxifying and antioxidant enzymes, such as glutathione reductase, glutathione peroxidase, glutathione *S*-reductase, catalase, and quinone reductase (Khan, Katiyar, Agarwal & Mukhtar, 1992; Valerio, Kepa, Pickwell & Quattrochi, 2001).

D. Anti-inflammatory activity

Although an inflammatory response is essential for the protection of organisms against microorganisms, chronic inflammation is believed to be an important factor in the promotion and progression of a number of cancers. Cyclooxygenase-1 and -2 (COX-1 and COX-2, (also called prostaglandin H synthetase PGHS)) are the major enzymes that convert arachidonic acid to prostaglandins and other downstream inflammatory factors. COX-1 is constitutively produced and is necessary for certain housekeeping functions of prostaglandins such as gastrointestinal cytoprotection, renal functions and vascular homeostasis (Goldenberg, 1999).

COX-2 expression, on the other hand, is induced by inflammatory stimuli and is considered as one of the indicators of chronic inflammation. A second enzyme called inducible nitric oxide synthase (iNOS) is expressed after exposure to various inflammatory stimuli such as lipopolysaccharide (LPS), tumor necrosis factor (TNF-α), or interleukin (IL)-1β, and has been shown to regulate expression of certain

cytokines and chemokines necessary for neutrophil trafficking during inflammation (Speyer et al., 2003). The anti-inflammatory activity of various flavonoids (e.g., quercetin, apigenin, tea catechins) are due to their inhibitory effects on COX-2 and iNOS (Mutoh, 2000; Raso, Meli, Di Carlo, Pacilio & Di Carlo, 2001). The consumption of low doses of specific COX-2 inhibitors, called coxibs, has also been shown to afford protection against colorectal carcinoma.

E. Growth inhibition

Various flavonoids have shown a growth inhibitory effect on several human cancer cell lines. In addition, it has been shown that flavonoids can alter cell signaling and cell cycle progression. For instance, tea flavonoids, particularly EGCG, possess inhibitory effects on signal transduction pathways induced by epidermal growth factor and platelet-derived growth factor, affecting downstream events such as angiogenesis (Wiseman, Mulder & Rietveld, 2001).

The inhibitory effect of the flavonoids, genistein, and quercetin on cell proliferation by the inhibition of protein tyrosine kinases(Akiyama et al., 1987; So, Guthrie, Chambers, Moussa & Carroll, 1996)as well as the effects on cell cycle arrest and apoptosis by apigenin, luteolin and quercetin in a p53-dependent mechanism are other evidence on the growth inhibitory effects of flavonoids in cancer cells (Plaumann, Fritsche, Rimpler, Brandner & Hess, 1996).

1.7.3 The Rebirth of Herbal Medicine

Carcinogenesis is a multistep process consisting of tumor initiation, promotion and progression (Dorai & Aggarwal, 2004). The process begins with cellular transformation, progresses to hyperproliferation and culminates in the acquisition of invasive potential, angiogenic properties and establishment of metastatic lesions (Hahn & Weinberg, 2002).

Various environmental carcinogens (such as cigarette smoke, industrial emissions, gasoline vapors), inflammatory agents (such as tumor necrosis factor (TNF) and H_2O_2), tumor promoters (such as phorbol esters and okadaic acid) are able to activate

carcinogenesis. The treatment of advanced cancer using natural medicines has drawn much attention recently (Vickers, 2000). Such natural medicines have been reported to serve as biological response modifiers by activating, increasing, and/or restoring the reactivity of immunological effector mechanisms that are involved in resistance to tumor growth and metastasis (Wasser & Weis, 1999; Werner & Jolles, 1996).

Population studies have shown that people in Southeast Asian countries have far lower risks of developing most cancers compared with those in North America, and it is considered that the consumption of foods such as garlic, ginger, cayenne, turmeric, soy, and cruciferous vegetables play a key role in this "chemoprevention." Chemoprevention involves the inhibition of multiple aspects of the tumorigenic process. (See Table1)

These dietary agents are believed to suppress the transformative, hyperproliferative and inflammatory processes that initiate carcinogenesis. These inhibitory influences may ultimately suppress the final steps of carcinogenesis, namely angiogenesis and metastasis (Dorai et al., 2004).

Since the intestines and colon are exposed to dietary components, prevention of cancer of the colon through the diet is an attractive option. Colorectal cancer is one of the leading causes of premature death worldwide. A multifaceted approach for the primary prevention of this disease, with emphasis on nutrition and diet has curried favor amongst many experts. During past decades, several naturally occurring compounds have been studied in terms of their antiproliferative and cancer preventive abilities in various in vitro and in vivo models of colorectal cancer. Significantly, these compounds are present in various popular and frequently consumed products such as wine grapes, teas, berries, peanuts etc and thereby provide opportunities to the entire population to tap into their beneficial effects. (Bravo, 1998; Hollman, Devries, Vanleeuwen, Mengelers & Katan, 1995; Lee et al., 1995).

Table 1. Comparison of cancer incidence in USA and India (Dorai et al., 2004)

Cancer	USA		India	
	Cases	Death	Cases	Death
Breast	660	160	79	41
Prostate	690	130	20	9
Colon/Rectum	530	220	30	18
Lung	660	580	38	37
Head & Neck SCC	140	44	153	103
Liver	41	44	12	13
Pancreas	108	103	8	8
Stomach	81	50	33	30
Melanoma	145	27	1.8	1
Testis	21	1	3	1
Bladder	202	43	15	11
Kidney	115	44	6	4
Brain, Nervous system	65	47	19	14
Thyroid	55	5	12	3
Endometrial Cancers	163	41	132	72
Ovary	76	50	20	12
Multiple myeloma	50	40	6	5
Leukemia	100	70	19	17
Non-Hodgkin Lymphoma	180	90	17	15
Hodgkin’s disease	20	5	7	4

1.7.3.1 Salicylates: Aspirin and the Willow Bark Family of Plants

One of the natural compounds have been used as therapeutic agents against colon cancer are the salicylates, a class of compounds which have been used for centuries as analgesic, antipyretic, and anti inflammatory drugs. Today they are also well known for their protective effects against colon cancer and cardiovascular disease (Patrignani, 2000). Aspirin (acetylsalicylic acid -ASA), the most commonly consumed non steroidal anti-inflammatory drug and its active ingredient salicylic acid (SA, 2-hydroxybenzoic acid) are two of the best known salicylates (Patrignani, 2000; Yu et al., 2002).

SA is a white crystalline solid that melts at 157-159°C and is only slightly soluble in cold water. The molecule is unusual in that it incorporates two distinctive and important functional groups: a phenol O-H (alcohol) and a carboxylic acid group COOH.

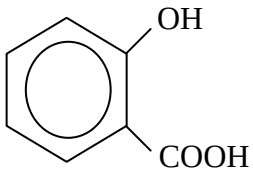


Figure 3: Salicylic acid showing the phenol and carboxylic acid groups.

Aspirin is the acetylated derivative of salicylic acid. As a pure compound, aspirin is a very stable solid, which melts near 135°C that can gradually hydrolyze to acetic acid and salicylic acid in the presence of moisture.

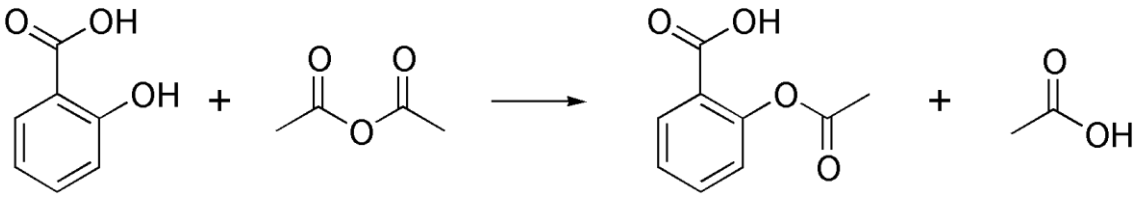


Figure 4: Formation of aspirin from salicylic acid.

The anti-inflammatory function of aspirin is thought to arise through the inhibition of the arachidonic acid metabolizing enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) leading to the inhibition of prostaglandin (PG) synthesis (Meade, Smith & Dewitt, 1993; Vane, 1978; Yu et al., 2002). Epidemiological studies have shown that patients under long term aspirin therapy have lower propensity to develop colorectal cancer (Fosslien, 2000; Giardiello et al.; Steinbach et al.) However, recent studies have suggested that ASA may act through some COX- independent pathways, therefore the mechanism of its anti-carcinogenic effects remains unclear (Law et al., 2000). However, its anti-proliferative effects on colon cancer remain unequivocal.

Since salicylic acid and other salicylates are naturally present in various fruits and vegetables, consuming these components in the daily diet might greatly reduce the risk of colorectal carcinoma (Paterson & Lawrence, 2001). Studies have shown that the concentration of salicylic acid in the serum of people consuming vegetables is greater than those who don't consume vegetables and those taking low-dose aspirin (Paterson et al., 2001).

The Willow family, Salix, consists of plants with notable amounts of endogenous salicylate compounds. Willow bark (Salix species) has been widely famed and used throughout the world since ancient times due to its active constituents, salicin, and its derivatives. Specifically the discovery of aspirin has diverted significant attention to this plant species (Barrett, Kiefer & Rabago, 1999; Cragg & Newman, 2001; Levesque & Lafont, 2000).

However, pharmacological studies have indicated that the fraction of total salicin is not adequate to make comprehensible the potency of willow bark. In addition, salicin and its metabolites do not have the acetylating potential of ASA. Therefore, there should be additional mechanisms to provide the anti-inflammatory potential of Willow Bark such as the participation of polyphenols and flavonoids as reported in some *in vitro and in vivo* studies. However, the identification of a specific compound or a combination of compounds, which can be responsible for the anti-inflammatory

action of Willow bark, remains to be clarified (Hostanska, Jurgenliemk, Abel, Nahrstedt & Saller, 2007a; Vane, 1978).

1.8 Conclusions

Owing to the harmful side effects of traditional chemotherapy drugs, as well as a trend towards more 'natural' and thereby 'safe' medication, a wide array of natural compounds derived from plants have been examined for anti cancer activity. Many of these plant extracts also possess high antioxidant activity and are able to scavenge reactive oxygen species and inhibit serious damage to cellular components (Ahmed, Rahman, Alam, Saleem, Athar & Sultana, 2000; Alam, Iqbal, Saleem, Ahmed & Sultana, 2000; Cadenas & Davies, 2000; Jayaprakasha, Mandadi, Poulose, Jadegoud, Gowda & Patil, 2007; Marnett, 2000; Sultana & Saleem, 2004; Tepe & Sokmen, 2007; Uchida, 2000).

However, caution should be exerted on the unregulated use of herbal medicines. Some side effects caused by contaminants and herb–drug interactions have been reported (Ernst, 1999). In the United States, herbal medicines are classified as dietary supplements and therefore are not subject to the stringent quality control that allopathic medicines are put through (Bent, 2008). It may therefore be beneficial to pursue the use of combinatorial therapies whereby the benefits of herbal medication as well as standard allopathic medicines are merged (Kanetkar, Singhal & Kamat, 2007).

The aim of our current study was to determine the antioxidant and salicin contents as well as anti proliferative potential of extracts from a species of Salix (*Salix aegyptiaca*) endogenous to the Middle East. To our knowledge this species of the plant has not been examined for it antioxidant profile or anti-carcinogenic effect to date. Our data indicates that extracts from *Salix aegyptiaca* have very high antioxidant capacities and salicin contents (Enayat and Banerjee, 2008 submitted) and at low doses are capable of inhibiting the proliferation of two colorectal cancer cell lines HCT-116 and HT-29. This species of plants is therefore an excellent source of antioxidants and can be consumed for its health promoting effects.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant selection

Plant collection was carried out according to published guidelines (N'Guessan, Bidie, Lenta, Weniger, Andre & Guede-Guina, 2007). Briefly, catkins, leaves and barks of *Salix aegyptiaca* were collected from Ghaene ghom, Iran, during the 2007 harvest season. The plant materials were identified morphologically at the herbarium of the Medicinal Plants Research Institute of Shahid Beheshti University of Tehran, Iran. The fresh catkins (15 kg), leaves (2 kg) and barks (1 kg) were air dried at room temperature for 1 week giving 1.5 kg, 500 g and 250 g dried catkins, leaves and barks respectively. The samples were then pulverized to a powder form using a steel blender (Sinbo, Istanbul, Turkey) and stored in a desiccator at 4°C in the dark until analysis.

2.1.2 Chemicals

Gallic acid, (+) catechin, epigallocatechin gallate, vanillin, myricetin, quercetin, rutin, caffeic acid, coumaric acid, Folin and Ciocalteu's phenol reagent, salicin acridine orange hydrate hydrochloride and DPPH were obtained from Sigma Chemical Co. (Taufkirchen, Germany). MTT Vybrant® cell viability assay kit (1000 assays) was obtained from Invitrogen (Carlsbad, CA, USA). Caspase-3/CPP32 Colorimetric Assay Kit was purchased from Biovision (CA, USA). All other chemicals used were purchased from Merck (Darmstadt, Germany) and were of chromatography grade. Distilled deionised water (ddH_2O) was used throughout the experiments.

2.2 Extract preparation

A total of eleven different extracts were prepared from different parts of the plant using the procedure of (Gezer et al., 2006) and (Kim, Jeong & Lee, 2003) with some modifications. Solvent selection was based on increasing polarity. The dried plant material from leaves, bark and catkins (10 g) were separately dissolved in cyclohexane (CycH) (non-polar), butanol (BuOH), ethanol (EtOH) and water (most polar) with the ratio of 1 to 10 (w/v) for barks and leaves and 1 to 40 (w/v) for catkins. As the catkins in dried powder form tended to float in all the solvents, it was necessary to use a higher volume of solvents in order to ensure the complete extraction of the active ingredients.

The samples were extracted by sonicating for 20 min in a bath sonicator (Bandelin Sonorex Model RK 100H, UK) followed by incubation at 30ºC with shaking at 150 rpm for 24h (Kim et al., 2003). The duration of sonication was maintained for short periods and the water of the bath was changed frequently to prevent any significant increase in the temperature of the extracts. Following this, they were filtered through a Whatman (No.1) filter paper and centrifuged at 5000 rpm for 10 min using an Eppendorf 5804R refrigerated benchtop centrifuge (Eppendorf, Germany). The procedure was repeated for second time by adding fresh solvents. The supernatants were then collected and concentrated in vacuo at 45ºC using a rotary evaporator (Buchi Rotavapor R-200, Essen, Germany), lyophilized and kept in the dark at -20ºC until analysis (Gezer et al., 2006; Kim et al., 2003)

2.3 Detection of antioxidant activity

Owing to the large number of extracts used in this study along with the necessary replications to make the data statistically relevant, all assays were scaled down to be accommodated in 96 well plates for absorbance measurements in a microplate reader. Flat bottom 96 well plates (Greiner, Frickenhausen, Germany, Catalog no: 655 101) were used in order to accommodate a maximum volume of 350μL without spillage.

2.3.1 DPPH Assay:

The antioxidant activities of all the extracts were evaluated by DPPH (2,2-diphenyl-1- picrylhydrazyl) free radical scavenging assay according to Gezer et al., (2006) and Okusa, et al., (2007) with some modifications. This assay is based on the ability of DPPH, a stable free radical, to be quenched and thereby decolourise in the presence of antioxidants resulting in a reduction in absorbance values (Gezer et al., 2006). Ten mg of the lyophilized powder for each of the eleven extracts was weighed in a precision balance (Adventurer Ohaus, Germany) and dissolved in 1ml methanol. This original stock solution (10 mg/ml) was further serially diluted with methanol to give a range of concentrations.

Twenty five microliters from these stock solutions were then added to 325µl of 0.005% methanolic DPPH solution in 96 well plates to give a final concentration range of 5 µg/ml – 120 µg/ml at room temperature. The DPPH solution was prepared freshly every day. The decrease in absorbance was measured at 490nm (Hwang et al., 2001) at an end point of 30 min and 1 h after incubation at 25°C in the dark in a Bio- Rad 680 microplate reader (BioRad, USA). The results were compared with quercetin and BHT as standards and methanol was used as a blank (Okusa et al., 2007). The percentage of DPPH free radical quenching activity was determined from the formula:

$$\text{DPPH Scavenging Effect (\%)} = \left(\frac{A_{DPPH} - A_{Extract}}{A_{DPPH}}\right) \times 100$$

Where ADPPH refers to the absorbance value at 490nm of the methanolic solution of DPPH and AExtract stands for the absorbance value at 490nm for the different extracts. A chart was generated for the percentage activity to quench DPPH free radical of each concentration (ranging from 5 µg/ml – 120 µg/ml) of the individual extracts using the equation above. The equation generated from this chart was used to determine the concentration of each extract required to quench 50% of the DPPH free radical activity (IC_{50} value). The antioxidant activity of the different extracts was also expressed in terms of quercetin equivalents (QE) and represented as mg of QE per gram of dried extract. Each sample was assayed at least six times.

2.3.2 Total phenol assay

Total phenolic constituents of the extracts were analysed by Folin–Ciocalteu method using gallic acid as standard as described by (Singleton, Orthofer & Lamuela-Raventos, 1999) with some modifications. For the preparation of gallic acid stock solution, 0.5 g of dry gallic acid was dissolved in 10mL of ethanol and diluted to 100 ml with ddH2O. Then 0, 1, 2, 3, 5, and 10 ml of the gallic acid stock solution was diluted to 100 ml with ddH2O in volumetric flasks giving an effective concentration range of 0, 50, 100, 150, 250, and 500 mg/l gallic acid.

The extracts (3.5 µl) from a 1mg/ml methanolic stock solution or standard solutions of gallic acid (3.5 µl) or ddH2O as blank were added to separate test tubes and mixed thoroughly with 276.5 µl ddH2O and 17.5 µl of Folin- ciocalteu reagent. After 8 min 52.5 µl of 7% Na_2CO_3 solution was added, and mixed thoroughly by pipetting. The final concentration of the extracts in each well was 10 µg/ml. The solutions were incubated at 20°C for 2 h and the absorbance versus blank (0 mg/l gallic acid) was read at 765 nm using a Bio-Rad 680 microplate reader (Bio-Rad, USA).

In addition, a methanol only sample was processed in the same manner to account for any background due to methanol. The total phenol content of the extracts was determined by comparing with a calibration curve of the gallic acid standard and represented as mg gallic acid equivalents (GAE) /g of dried samples. Each sample was assayed at least six times.

2.3.3 Total flavonoid assay

The total flavonoid content in the extracts was determined by aluminum colorimetric assay (Zhishen, Mengcheng & Jianming, 1999) with some modifications. A standard solution of (+)catechin at different concentrations (20, 40, 60, 80 and 100 mg/l) was prepared by dissolving (+)catechin in ddH2O. The extracts (35 µl) from a 1mg/ml stock solution or standard (+)catechin solutions (35µl) or ddH2O (as blank) were mixed thoroughly by pipetting with 140 µl ddH2O in a 96 well plate. Then 10.5 µl 5% sodium nitrite (NaNO2) was added. The mixture was incubated for 5 min at 25°C and 10.5 µl of 10% aluminium chloride ($AlCl_3$) was added to and then 6 min later, 70

µl 1 M NaOH was added to the wells. The total volume was made up to 350 µl by adding ddH2O. The absorbance was read at 490 nm in a BioRad 680 microplate reader (BioRad, USA). ddH2O was used as a blank. Additionally, a methanol only sample was processed in the same manner to account for any background due to methanol. The total flavonoid content was expressed as mg of catechin equivalents (CE)/g of dried samples. Each sample was assayed at least 6 times.

2.4 Identification of salicin and individual phenolic compounds by HPLC

2.4.1 Chromatographic conditions

Salicin and phenolics compounds in the extracts were analyzed by high performance liquid chromatography (HPLC) on a Varian ProStar HPLC with a Varian 330 PDA detector (Varian, Palo Alto, CA, USA). Chromatographic data were acquired and processed using the Varian Star Workstation accompanying the HPLC equipment. The chromatographic conditions for the determination of salicin was according to the procedure by (Minakhmetov, Onuchak, Kurkin, Zapesochnaya & Medvedeva, 2002) and (Poblocka-Olech, van Nederkassel, Heyden, Krauze-Baranowska, Glod & Baczek, 2007), while the other phenolic compounds were detected according to (Garcia-Falcon, Perez-Lamela, Martinez-Carballo & Simal-Gandara, 2007) with some modifications (Table 2).

Table 2: shows the optimized chromatographic conditions for the determination of salicin, phenolic acids and flavonols.

Chromatographic conditions	
Analytical column	*Phenolic compounds* Varian pursuit C18 (150 4.6 m, 5 μm) *Salicin* Varian Intersil ODS3 (150 x 4.6 m, 5 μm)
Injected volume	100 μl
Mobile phase	*Phenolic compounds* A: (Formic acid in water, 2.5% v/v) B: (Methanol) 0-7 min: 100:0 (elution step) 7-42 min: 80:20 (elution step) 42-47 min: 80:20 (elution step) 47-72 min: 60:40 (elution step) 72-73 min: 100:0 73-75 min: 100:0 (conditioning step) *Salicin* A: Water and trifluoroacetic acid (TFA) (100:0.05 v/v) B: acetonitrile Linear gradient 0-30min: 97% to 52% for A 0-30 min: 3% to 48% for B
Flow rate	1 ml/min
Temperature	35°C for phenolic compounds 30°C for salicin
Detection conditions	
Scanning	200-600 nm
Detection wavelength	λ 268 : Salicin λ 280 : gallic acid, catechin, epigallocatechin gallate (EGCG), Vanillin λ 320 : caffeic acid, p-coumaric acid λ 360 : rutin, myricetin, quercetin

2.4.2 Preparation of extracts and standards

The ethanolic extracts of bark and catkins and the water extract of leaves possessing the highest total phenolic and flavonoid contents were selectively analyzed to further identify and quantify a range of phenolic and flavonoid compounds by HPLC. The dried ethanolic extracts of bark and catkins and the water extract of leaves were dissolved in methanol at a concentration of 10 mg/ml, centrifuged at 14,000 rpm (11481 x g) for 10 min, filtered through a 0.45μm filter and injected in the column via an automated injection system. For salicin, a standard curve was generated by duplicate injections of different concentrations (10-0.5 mg/ml) of standard salicin in methanol. The standard compounds selected for the identification of phenolic compounds were gallic acid, vanillin, caffeic acid, coumaric acid, and the flavonoids catechin, epigallocatechin gallate (EGCG), rutin, myricetin and quercetin. A standard curve for each compound was generated by duplicate injections of a serial dilution of a stock solution of 1mg/ml in methanol to give a range of different concentrations between 2-100 μg/ml.

2.4.3 Determination of salicin content

Since the Willow family of plants is known for their salicin content and potential therapeutic benefits, we determined the salicin level in bark, leaves and catkins extracted in all four solvents (water, ethanol, butanol and cyclohexane). Each extract was injected at least 3 times and the retention time of salicin was ~10 min.

Since the retention time of peaks can alter in discrete but small units between runs, a standard salicin sample (1 mg/ml) was injected after every sample injection to confirm the retention time of the salicin peak. In addition, the extracts were spiked with pure standard salicin for each run in order to confirm the retention time and help in the identification of the salicin peak in the extracts.

2.4.4 Determination of phenolic compounds

The phenolic compounds were identified on the basis of a comparison of their retention time with that of authentic standards. Each sample was injected at least 3 times and a mix of the standards was injected after every sample injection to confirm

the retention time of each identified peak. In addition, for some phenolic compounds the extracts were spiked with the pure standard compound in order to further confirm the retention time and identity of the compound.

2.5 Effects of S. aegyptica extracts on colon cancer cell lines:

Since the long term use of aspirin and other NSAIDs is associated with anti-carcinogenic effect, the ethanolic extracts from catkins, leaves and bark of *Salix aegyptiaca* with strong antioxidant activity and high phenol and flavonoid contents were assayed for possible effects on cellular proliferation using two representative human colon cancer cell lines: HT-29 which expresses COX-2 and HCT-116 which lacks the COX-2 transcript.

HT-29 and HCT-116 cell lines were grown in RPMI 1640 media supplemented with 10% fetal calf serum (v/v) 2mM L-glutamine, penicillin/streptomycin 1%. The cultures were maintained at 37ºC in a humidified atmosphere of 95% air and 5% CO_2 according to ATCC guidelines.

2.5.1 Preparation of the extracts in media:

Lyophilized ethanolic extracts from catkins, leaves and bark of *S. aegyptiaca* were dissolved in DMSO to give a stock solution of 50 mg/ml. The stock solution was then diluted in complete RPMI1640 medium to obtain a concentration of 5 mg/ml. This solution was filtered through 0.2 μm syringe filter, and further diluted with sterile complete RPMI 1640 medium to get a range of dilutions between 50μg/ml and 350μg/ml. The final DMSO concentration was kept at below 0.5%.

2.5.2 MTT assay:

The growth inhibitory effects of ethanolic extracts from leaves, bark and catkins of *S. aegyptiaca* on HCT-116 and HT-29 cell lines were measured by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay according to manufacturer's instructions. This assay measures the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan by a dehydrogenase activity in the mitochondria of viable

cells. For the assay, both cell lines were seeded in 96-well plates in triplicate separately at a density of 10^4 cells/well in the absence or presence of different dilutions of the extracts. The effect of the extracts on the cell lines as a function of both dose and time were assayed.

To determine the effect of the extracts as a function of time, 10,000 cells/well (HT-29 and HCT-116) were plated in 96 well plates and incubated with extracts at a final concentration of 50 μg/ml for 24, 48 and 72 h at 37°C in a humidified atmosphere with 5% CO_2. After the incubation time, the medium was changed and 10 μl 12M MTT was added to each well and incubated at 37°C for 4 h. One hundred microliters per well of solubilization solution containing 10% SDS in 0.01 M HCL was added into each well and incubated for a further 18h at 37°C in a humidified incubator. The absorbance of each well was read at 570nm in a BioRad 680 microplate reader (BioRad, USA). The medium with MTT reagent was used as a blank. Each assay was carried out in triplicate and the experiment was repeated at least 3 times.

To determine the effect of the extracts as a function of their concentration, 10,000 cells/well were seeded in 96-well plates and incubated with different dilutions of extracts from 50 □ g/ml to 350□ g/ml for 48h. After the incubation time, the medium was changed and incubated with the MTT reagent as described above. The absorbance of each well was read at 570 nm in a microplate reader.

The growth inhibitory percentage (GI%) for each concentration of the extracts was calculated as follows:

$$\frac{\text{Mean absorbance of treated cells}}{\text{Mean absorbance of untreated cells}} \times 100$$

The concentration of substances that reduced cell growth by 50% (GI50) was calculated from the dose–response relationship by regression analysis using Microsoft Excel computer software. Each assay was carried out in triplicate.

2.5.3 Apoptosis assays

Inhibition of apoptosis is one of the key features of tumor cells and the progression of cancer. Therefore the extracts of *Salix aegyptiaca* were examined for their possible effects on induction of apoptosis by two different methods, acridine orange staining assay and caspase 3 activity assay.

2.5.3.1 Acridine orange staining assay:

Apoptotic activity of extracts was determined by acridine orange (AO) staining assay using the acridine orange dye. AO is a metachromic dye which differentially stains double-stranded (ds) and single-stranded (ss) nucleic acids. When AO intercalates into dsDNA it emits green fluorescence upon excitation at 480-490 nm. On the other hand it emits red when interacting with ssDNA or RNA. Therefore, this assay is based on the difference in the emission of fluorescence when AO interacts with normal and apoptotic cells. Since chromatin condensation is an early event of apoptosis, condensed chromatin is much more sensitive to DNA denaturation than normal chromatin. Therefore, if RNA is removed by pre-incubation of cell suspension with RNase A and DNA is denatured in situ by exposure to HCL shortly before AO staining; apoptotic cells (which have larger fraction of DNA in the denatured form) display an intense red fluorescence and a reduced green emission when compared to non-apoptotic interphase cells.

Method:

In order to prepare the staining solution, 6 mg/ml acridine orange was mixed with 90 ml 0.1 M citric acid followed by 10 ml 0.2 M Na_2HPO_4 (pH 2.6).

HCT-116 cells (1×10^6) were seeded separately in a 6-well plate. After 24 hours incubation the cells were treated with 250 and 500 μg/ml salicin and 250 μg/ml ethanolic extract of bark, leaves and catkins separately and incubated at 37°C for 48 hours. The concentration of the extracts was selected according to the average concentration of extracts required for 50% inhibition in cell proliferation after 48 hour treatment as observed by MTT. Sodium butyrate (NaBt, 5 mM) was used as a positive control. Cells that were incubated without any extracts served as the

negative control. The same procedures were done for HT-29 colon cancer cells. After 48 hours the supernatant media including dead cells from each well were transferred into separate sterile polypropylene tubes. The rest of the cells which were still attached to the wells were trypsinized by 500 μl trypsin/EDTA for 5 min followed by adding complete medium to terminate the trypsin reaction. These cells were added into previously collected cells in each tube. The cells were centrifuged at 200 x *g* for 5 min, the cell pellets were collected and further washed with PBS and centrifuged at 200 x *g* for 5 min and then resuspended again in 1 ml PBS. The cells were fixed by adding 9 ml 1% paraformaldehyde in PBS and incubated for 15 min on ice. The fixed cells were centrifuged at 200 x *g* for 5 min and the cell pellets were resuspended in 5 ml PBS, followed by centrifugation at 200 x *g* for 5 min. The cells were then resuspended in 1 ml PBS following which 9 ml 70% (v/v) ethanol was added and stored for at least 4h at 4°C until analysis.

On the day of analysis the cell suspensions were centrifuged at 200 x *g* for 5 min and the cell pellets resuspended in 1 ml PBS. RNAse A solution (20μl of 1mg/ml) was added to the cell suspensions followed by 30 min incubation at 37°C. Later the cells were centrifuged at 200 x *g* for 5 min and resuspended in 0.2 ml PBS. At room temperature 0.5 ml 0.1 M HCL was added into each cell suspension. After 30 to 45 s, 2 ml of acridine orange solution prepared as mentioned above was added to the cells. The cell suspension (10μl) was pipetted onto a glass slide and observed under a confocal microscope (Zeiss LSM 510, METU Molecular Biology and Biotechnology Central Laboratory) at 480-490 nm. At least 1000 cells were counted and the number of apoptotic cells was determined on the basis of their color.

The fold increase in the number of apoptotic cells was calculated according to the ratio of the mean of the number of apoptotic cells in each sample to the mean of the number of apoptotic cells in control. The experiment was repeated at least 4 times.

2.5.3.2 Caspase 3 activity assay:

Caspases are a group of proteases, which play a crucial role in induction of apoptosis and inflammation. Caspase 3 is one of the caspases that is activated early during programmed cell death. Therefore the effects of extracts from *Salix aegyptiaca* in the induction of apoptosis were determined by measuring the activity of caspase 3 in both HCT-116 and HT-29 cell lines using Caspase-3/CPP32 Colorimetric Assay Kit (Biovision, CA, USA). The assay is base on spectrophotometric detection of the chromophore p-nitroanilide (pNA) light emission after cleavage from the labeled susbtrate DEVD-pNA.

Method:

The procedure was carried out according to manufacturer's guidelines. Briefly, 2 $\times 10^6$ cells were seeded in 6-well plates separately in each well. After 24 hours the cells were treated separately with 250 μg/ml ethanolic extracts of catkins, leaves and bark, and incubated at 37°C for 24 hours. Salicin and ASA (250ug/ml) were used along with the other extracts.

After 24 hour incubation of the cells with the extracts, the supernatants containing dead cells were removed and the attached cells were trypsinized and collected. The cell suspensions were centrifuged at 200 x *g* for 5 min. The cell pellets were washed with PBS and centrifuged at 200 x *g* for 5min following which the supernatant PBS was completely removed. The cell pellets were then resuspended in 50 μl of chilled lysis buffer (included in the kit) and incubated on ice for 10 min. Then the cell suspensions were centrifuged at 10,000 × *g* for 1 min. The supernatants containing cytosolic extracts were transferred to fresh tubes. The cytosolic fraction (10μl) from each sample was used for protein measurement using the modified Bradford method (Bradford, 1976) and the remaining stored at -80°C for future use.

On the day of analysis 200 μg protein was diluted and added up to 50 μl cell lysis buffer for each assay. Then 2x reaction buffer (50 μl) containing 10 mM DTT (Dithiothreitol) were added to each sample. Dithiothreitol is a reducing agent used to reduce the cysteine residue of caspase 3 which is essential for proteinase activity of

the protein (Baker, Dos Santos & Powis, 2000; Cleland, 1964). Later 5 μl 4 mM DEVD-pNA substrate (200 μM final concentration) was added to the mixture and incubated at 37°C for 2 hours. The samples were further diluted and added up to 1 ml by using a dilution buffer (included in the kit) and read at 400- 405nm in a Bio-Rad 680 microplate reader. A mixture of 50 μl cell lysis buffer and 50 μl 2x reaction buffer containing 10 mM DTT was used as a blank. Each assay were repeated at least 3 times.

2.6 Statistical Analyses

Data analysis and graphing was performed using the GraphPad Prism 5 software package (La Jolla CA, USA). Specific analysis for each experiment is indicated in each figure legend. In most cases, the mean of at least six experiments is plotted together with the standard deviation. Statistical analysis between experimental results was based on Spearman's non parametric correlation and Student's t test. Significant difference was statistically considered at the level of $P<0.05$.

CHAPTER 3

RESULTS

3.1 Results

3.2 Determination of antioxidant activity

Free radicals are one of most important causes of intracellular damages leading to several types of cancer. Antioxidants with the ability of scavenging and detoxifying these reactive compounds might introduce a protective role against cancer. In addition, since it is now accepted that antioxidants participate in several signal transduction pathways, more attention has been focused on antioxidants as therapeutic agents against cancer. Therefore in this study the antioxidant activity of extracts was determined in order to investigate any possible link between the antioxidant capacity and antiproliferative effects of extracts possessing high antioxidant potency. Moreover, according to recent reports the salicin content of willow bark is not sufficient to be the unique responsible factor for anti-proliferative effects of willow; furthermore it doesn't possess the acetylating potential of spirin (Hostanska et al., 2007a; Vane, 1978). Therefore there should be additional factors such as flavonoids and polyphenols participating in anti-proliferative potential of willow bark.

3.2.1 DPPH (1, 1-diphenyl -2-picryl hydrazyl) assay

The DPPH radical scavenging activity of the different extracts from catkins, leaves, bark of *S. aegyptiaca* was assayed as described in Materials and Methods (Section 2.3.1). An extract with high antioxidant activity was capable of quenching the DPPH free radicals at a much lower concentration. The results shown in Figure 5 indicate that the highest antioxidant activity was observed in the ethanolic extract of bark

with an IC_{50} value of 19 ± 3 μg/ml, while the lowest activity was shown by the bark extract in cyclohexane with an IC_{50} value of 319 ± 4 μg/ml. Radical scavenging activity (in μg/ml) of the remaining extracts were as follows: Leaves (water) 30 ± 1 > Bark (BuOH) 30 ± 6 > Bark (water) 40 ± 2 > Leaves (EtOH) 44 ± 1 > Catkin (EtOH) 49 ± 4 >Catkin (BuOH) 52 ± 0.2 >Leaves (BuOH) 63 ± 2 > Catkin (water) 113 ± 7 > Leaves (Cyc.H) 142 ± 6. The solvents in parenthesis indicate the solvent in which the plant parts were initially extracted. The IC_{50} values of the standards were 3.1 and 26 μg/ml for quercetin and BHT respectively.

When the DPPH free radical quenching data is represented as quercetin equivalents per gram of dried extracts, the ethanolic extract of the bark with 169 ± 28 mg QE equivalent/g dried sample showed the highest amount of quercetin equivalents. The milligram quercetin equivalents/g of dried extracts of other samples were as follows: bark (BuOH) 105± 20 > leaves (water) 103 ± 4 > bark (water) 78 ± 4 > leaves (EtOH) 70 ± 2 > catkin (EtOH) 64 ± 5 > catkin (BuOH) 60 ± 0.3 > leaves (BuOH) 49 ± 1 > catkin (water) 29 ± 2 > leaves (Cyc.H) 22 ± 1> bark (Cyc.H) 10 ± 0.1 (Figure 6 and Table 3).

The quercetin equivalence per gram of dried extracts was calculated according to the formula:

$$\frac{IC50\ value\ of\ quercetin}{IC50\ value\ of\ extract} \times 1000$$

Owing to the phenolic structure of salicin, we also examined the free radical quenching ability of purified commercial salicin by the DPPH free radical scavenging assay. However, commercial salicin as well as acetyl salicylic acid did not show any free radical quenching activity (data not shown). When a statistical correlation analysis was conducted between the salicin content and the antioxidant activity of the extracts we could not detect any significant correlation (Spearman's r =0.4727).

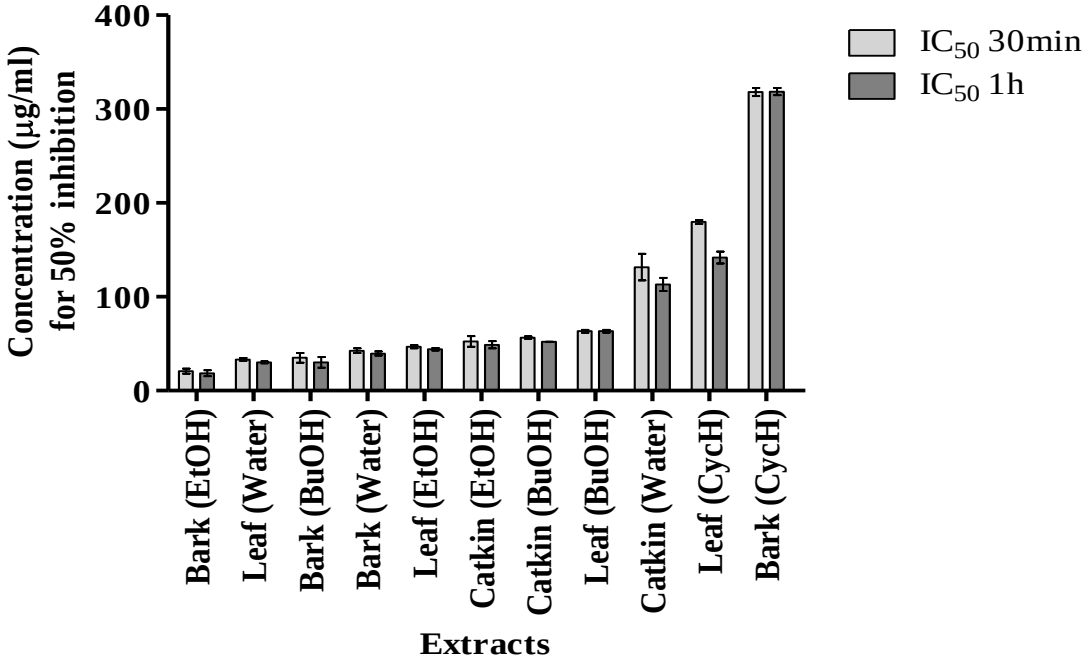


Figure 5:DPPH free radical scavenging assay showing the concentration of extracts necessary for 50% inhibition of the free radical activity of DPPH at two time points (30 min and 1h). The data are displayed with mean ± standard deviation of six replicates. The names in parentheses indicate the solvent into which the parts of the plants were initially extracted.(Ethanol: EtOH, Butanol: BuOH, Cyclohexane: CycH)

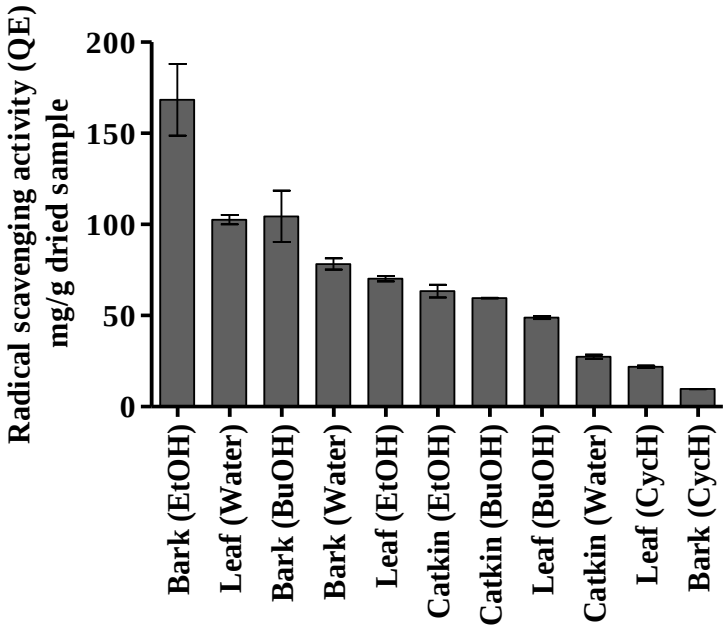


Figure 6:DPPH free radical quenching represented as mg of quercetin equivalents per gram of dried extracts capable of 50% inhibition of radical activity. The data are displayed with mean ± standard deviation of six replicates. The names in parentheses indicate the solvent into which the parts of the plants were initially extracted.

3.2.2 Determination of total phenol

The total phenolic content of different extracts from catkins, leaves, bark of *S. aegyptiaca* was assayed by the Folin–Ciocalteu method using gallic acid as standard (y = 0.0666x + 0.0634, R2 = 0.9905) as described in Materials and Methods (Section 2.3.2). The data presented in Figure 7 and Table 3 indicates that the highest total phenol content of 212 ± 4 mg GAE equivalents/g of dried sample was obtained in the ethanolic extract of bark while the lowest total phenol content of 4 ± 1 mg GAE equivalents/g of dried sample was obtained in cyclohexane extract of bark. The samples where the extracts were replaced with methanol only did not show any background absorbance due to methanol. The total phenol content of the other extracts (in GAE equivalents/g of dried sample) were as follows: Bark (BuOH) 211 ± 3 > Bark (Water) 139 ± 1> Leaves (water) 163 ± 3> Catkin (EtOH) 107 ± 3 > Catkin (Water) 86 ± 1, Catkin (BuOH) 81 ± 1, Leaves (EtOH) 64 ± 1, Leaves (BuOH) 36 ± 1 and Leaves (Cyc.H) 19 ± 1. The solvents in parenthesis indicate the solvent in which the plant parts were initially extracted.

3.2.3 Determination of total flavonoids

The total flavonoid content of different extracts from catkins, leaves, bark of *S. aegyptiaca* was assayed by aluminium colorimetric assay as described in Materials and Methods (Section 2.3.3). (+)Catechin was used as a standard (y = 0.0043x + 0.0305, R2 = 0.9948) and the total flavonoid content was expressed as mg of catechin equivalents (CE)/g of dried samples. The data presented in Figure 7 indicates that the highest flavonoid content of 479 ± 63 mg CE /g of dried samples was observed in the ethanolic extract of bark and the lowest content was observed in the cyclohexane extract of the leaves (2 ± 1 mg CE/g of dried samples). The samples where the extracts were replaced with methanol only did not show any background absorbance due to methanol. The total flavonoid contents of the other extracts (expressed in mg CE/g dried sample) were as follows: Bark (BuOH) 419 ± 4> Catkin (EtOH) 351 ± 2> Leaves (water) 280 ± 4> Bark (water) 243 ± 10> Leaves (EtOH) 165 ± 3> Catkin (BuOH) 152 ± 6> Leaves (BuOH) 125 ± 23> Catkin (water) 68 ± 15> Bark (Cyc.H) 73 ± 5 (Table 3).

Statistical analyses of the correlation between radical scavenging potential of the extracts and their total phenol and flavonoid contents have been represented in Figure 8 A and B.

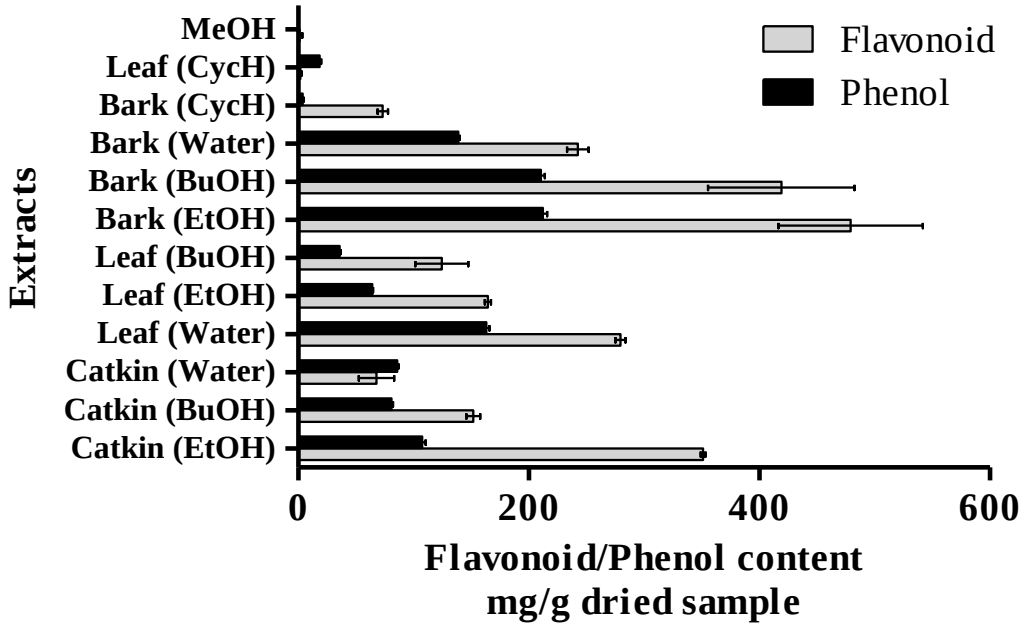
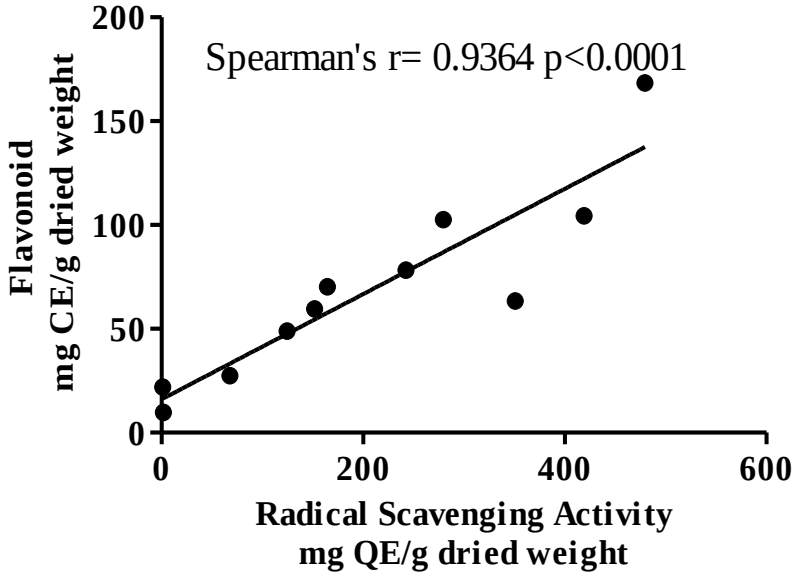
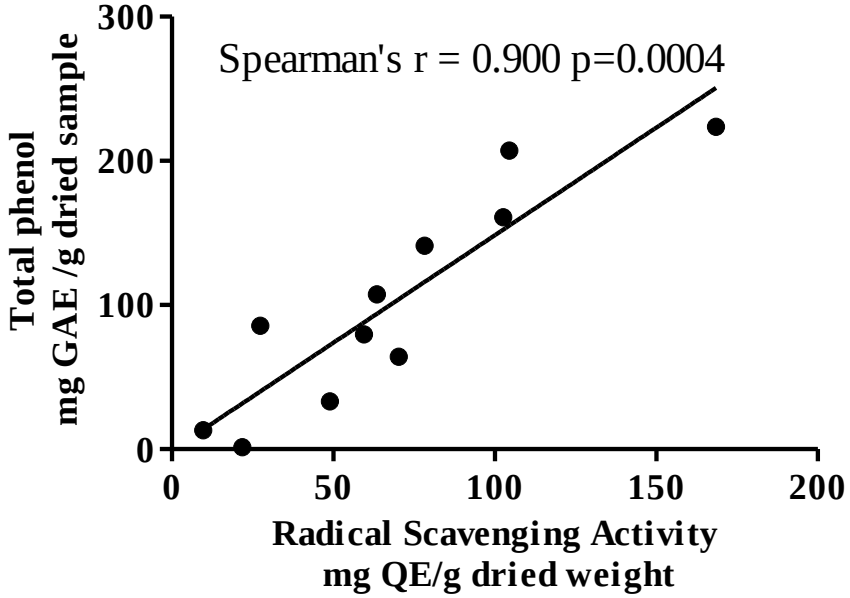


Figure 7: Total phenol and flavonoid content of various extracts of *S.aegyptiaca* expressed as gallic acid and catechin equivalents per gram of dried sample respectively. The data are displayed with mean ± standard deviation of six replicates. The names in parentheses indicate the solvent into which the parts of the plants were initially extracted.



(A)



(B)

Figure 8:(A): Correlation between flavonoid content (expressed as mg of catechin equivalents) and radical scavenging potential of extracts capable of 50% inhibition of radical activity expressed as mg of quercetin equivalents per gram of dried extracts. The chart indicates good correlation at a high significance level ($p<0.0001$) between flavonoid content and radical scavenging capacity of the extracts. B: Correlation between phenolic content and radical scavenging potential of extracts capable of 50% inhibition of radical activity expressed as mg of quercetin equivalents per gram of dried extracts. The chart exhibits high correlation ($p=0.0004$) between phenol contents and radical scavenging capacity of the extracts.

Table 3: Antioxidant activity, contents of total phenols and flavonoids and salicin in the different extracts of *S. aegyptiaca*. QE= quercetin equivalents GAE= gallic acid equivalents and CE = catechin equivalents. Numbers in parentheses represent the standard deviation.

Samples	**Antioxidant activity** (mg QE/g dried sample)	**Total phenol** (mg GAE/g dried sample)	**Total Flavonoid** (mg CE/g dried sample)	**Salicin content** (mg/ml of extract)
Catkin BuOH	60 (± 0.3)	81 (± 1)	152 (± 6)	0.9 (± 0.1)
Catkin EtOH	64 (± 5)	107 (± 3)	351 (± 2)	0.2 (± 0.002)
Catkin Water	29 (± 2)	86 (± 1)	68 (± 15)	1.8 (± 0.1)
Leaf BuOH	49 (± 1)	36 (± 1)	125 (± 23)	1.3 (± 0.03)
Leaf EtOH	70 (± 2)	64 (± 1)	165 (± 3)	0.7 (± 0.002)
Leaf Water	103 (± 4)	163 (± 3)	280 (± 4)	1.4 (± 0.02)
Leaf Cyc.H	22 (± 1)	19 (± 1)	2 (± 1)	0.07 (± 0.01)
Bark BuOH	105 (± 20)	211 (± 3)	419 (± 64)	0.2 (± 0.02)
Bark EtOH	169 (± 28)	212 (± 4)	479 (± 63)	3.1 (± 0.04)
Bark Water	78 (± 4)	139 (± 1)	243 (± 10)	0.07 (± 0.01)
Bark Cyc.H	10 (± 0.1)	4 (± 1)	73 (± 5)	0.04 (± 0.01)

3.3 Determination of salicin content by HPLC

For centuries, the bark extracts from Willow family have been used throughout the word as antipyretic, analgesic and anti-inflammatory drug. These effects have been referred to salicylate compounds including salicin, present in the extracts of this plant species and are famed as therapeutic agents against colon cancer. It is hypothesized that salicylates introduce their antiproliferative and anticarcinogenic effects by inhibition of arachidonic acid metabolizing enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) leading to the inhibition of prostaglandin (PG) synthesis

(Yu, et al*;* Gilman, et al., 1990; Moncada and Vane., 1979; Meade et al., 1993., Xu, et al., 1999).

In this study, the salicin content of the extracts was determined by using high performance liquid chromatography as described in Materials and Methods (Section 2.4). A representative figure of standard salicin has been shown in Figure 9(A). Analysis of the chromatograms (Appendix A.1) and the quantification of salicin shown in Figure 10 indicate the presence of notable amounts of salicin in various extracts of bark, catkins, and leaves of the plant. Based on area under the curve calculations and comparison with a standard curve of different concentrations of salicin, the highest salicin content was obtained in the ethanolic extract of bark (3.1 ± 0.04 mg/ml) followed by the water extract of catkins (1.8 ± 0.1 mg/ml) and water and butanolic extract of leaves with 1.4 ± 0.02 mg/ml and 1.3 ± 0.03 mg/ml respectively (Table 3). This was expected, since the bark of Salix species, particularly the ethanolic extracts, are known to harbour high amounts of salicin (Guvenc, Arihan, Altun, Dinc & Baleanu, 2007; Young, 2004). The salicin content of each extract was calculated based on 10 mg/ml concentration of original stock solutions.

(A)

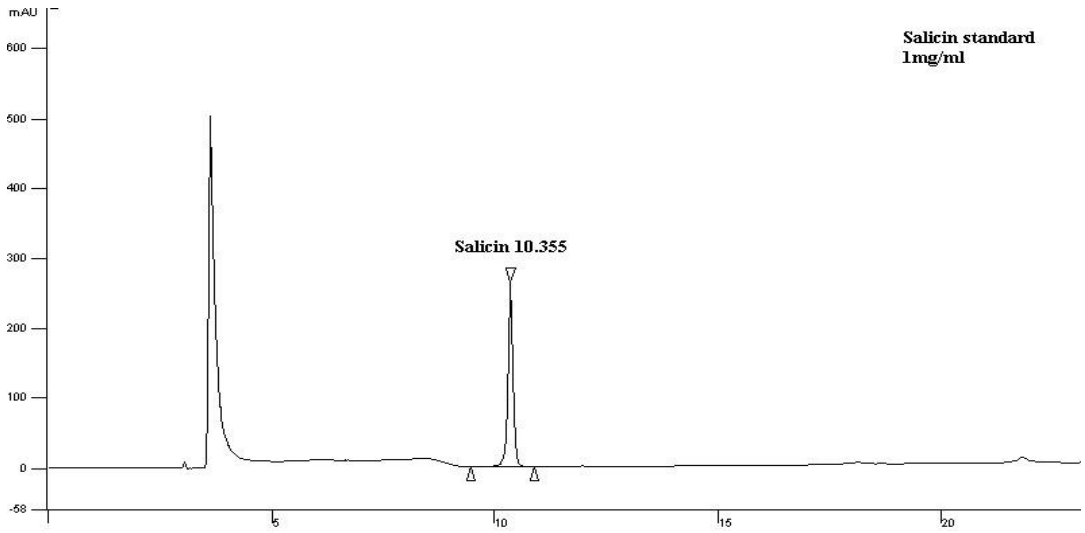


Figure 9(A): Chromatogram of the standard salicin (A). Chromatograms (B-L) have been presented in appendix A.1)

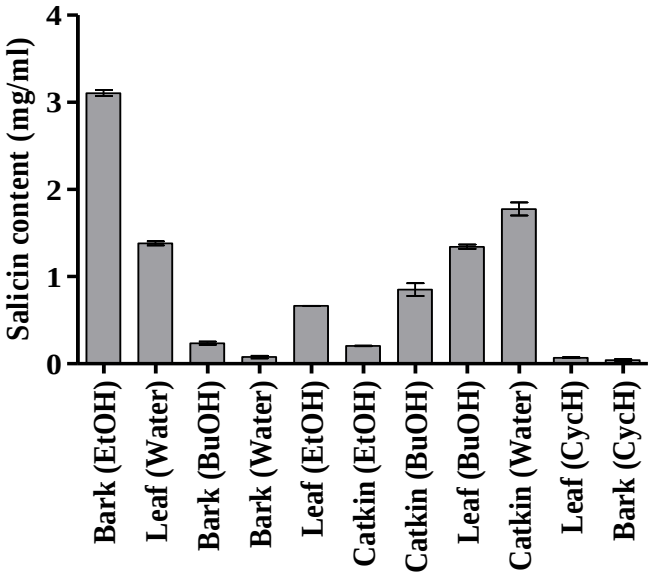


Figure 10: Salicin content (in mg/ml) in various extracts of catkins, leaves, and bark of *S. aegyptiaca* as detected by HPLC. A standard curve of different concentrations of salicin was generated as described in Materials and Methods (Section 2.4) and the amount of salicin was determined based on area under the curve calculations. The names in parentheses indicate the solvent into which the parts of the plants were initially extracted.

3.3.1 Determination of phenolic and flavonoid compounds by HPLC

Although willow extracts have been traditionally used as anti-inflammatory compounds for their salicin content, our data and previous studies on some other species of Salix indicate that these plants are also rich sources of phytochemicals and the antioxidant potential of the flavonoids and phenols may contribute to the beneficial effects seen with the consumption of commercial willow extracts (Nahrstedt, Schmidt, Jäggi, Metz & Khayyal, 2007).

The phenolic compounds; gallic acid, p-coumaric acid, vanillin and caffeic acid and flavonoids; catechin, epigallocatechin gallate (EGCG), rutin, quercetin and myricetin were identified and quantified in ethanolic extract of catkins, leaves and bark by HPLC (Table 4, for chromatograms please see Appendix A.2). Among the compounds identified, catechin (83.3 ± 3.9 mg/l) and myricetin (82.1 ± 9.5 mg/l) were the most abundant polyphenols in the water extract of leaves. Myricetin (58.7 ± 4.2 mg/l) and rutin (45.9 ± 2.56 mg/l) were the predominant flavonols in the ethanolic extract of bark, and catechin (9.9 ± 0.12 mg/l) and epigallocatechin gallate (9.8 ± 0.06 mg/l) were the major flavonols identified in the ethanolic extract of catkins. The chromatograms of the standards have been presented in Figure 11 (A-C).

(A)

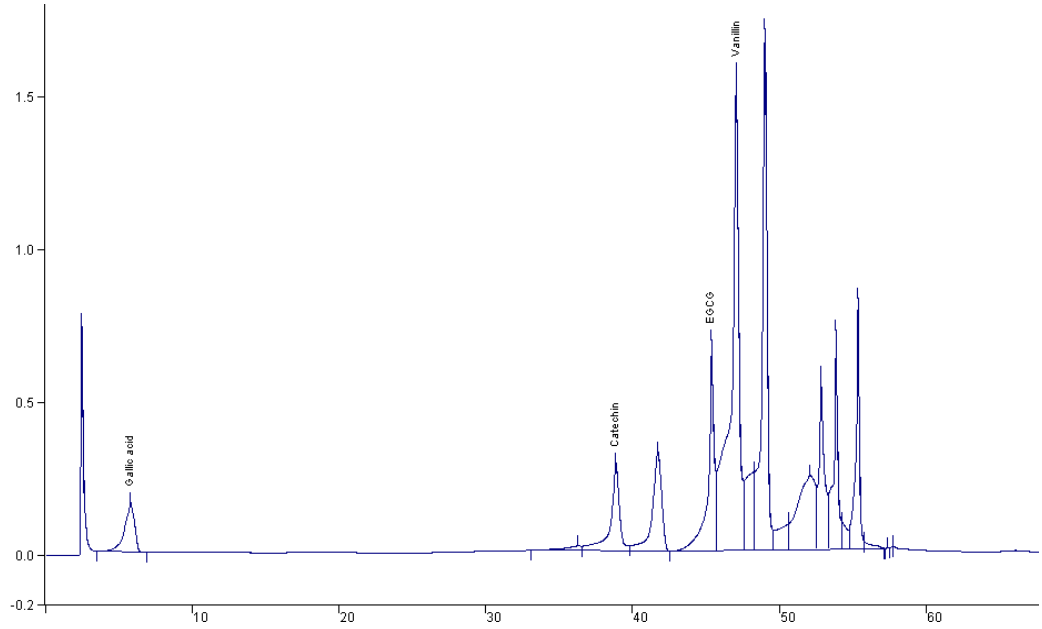
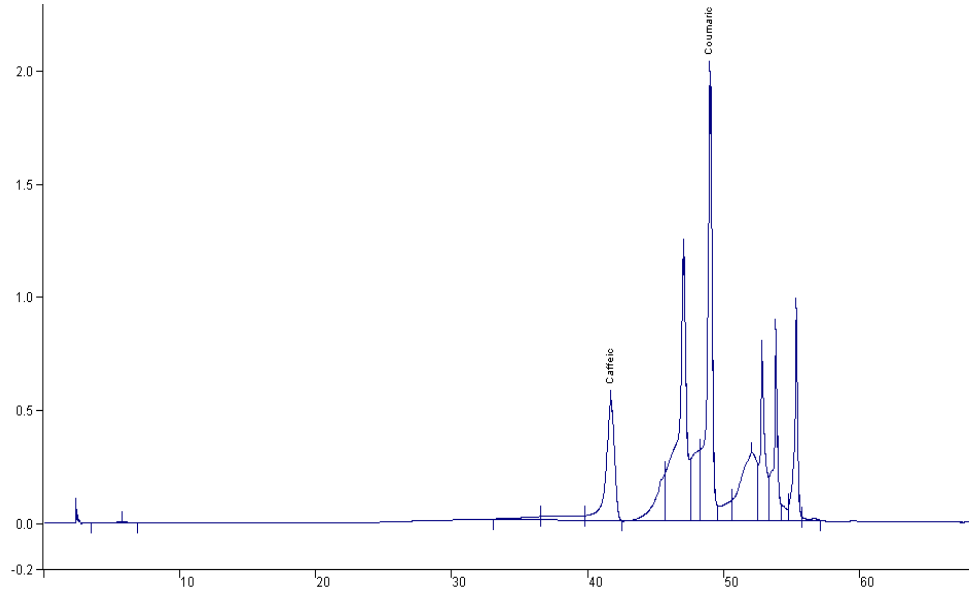


Figure 11(A): Chromatograms of the standards A: gallic acid, catechin, EGCG, vanillin (280 nm). (B and C): Chromatograms of the standards B: caffeic acid, p-coumaric acid (320 nm), C: myricetin, rutin, quercetin (360 nm).

(B)



(C)

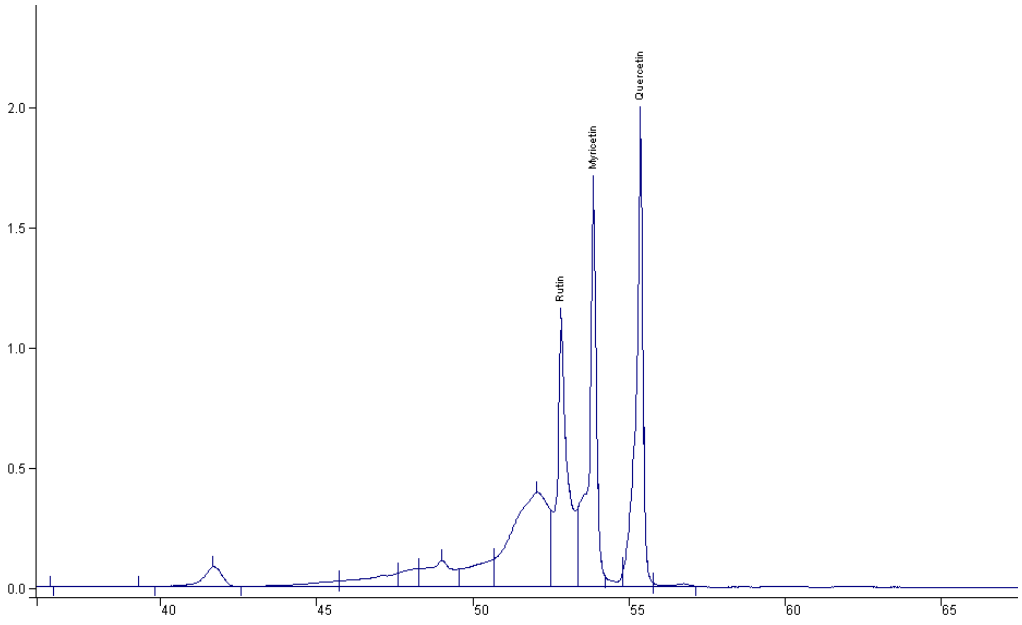


Fig.11: Continued

Table 4: The phenolics and flavonols present in different extracts of *Salix aegyptiaca.* The numbers in parenthesis represent mean and standard deviation of at least 3 experiments.
n.d: not detected.

Category	Compound	Retention time (min)	Catkins (mg/l)	Leaf (mg/l)	Bark (mg/l)
Phenolics	Gallic acid	5.1 - 5.8	0.5 (± 0.03)	0.4 (± 0.04)	6.9 (± 0.20)
	Caffeic acid	41.7 – 45.2	5.27 (± 0.1)	1.5 (± 0.1)	0.64 (± 0.1)
	Vanillin	47.1 – 49.4	3.4 (± 0.49)	5.2 (± 0.47)	15.3 (± 1.30)
	p-Coumaric acid	48.9 – 50.7	1.8 (± 0.32)	9.2 (± 0.81)	8.03 (± 0.4)
Flavonols	Myricetin	53.0 – 56.6	n.d.	82.1 (± 9.5)	58.7 (± 4.2)
	Catechin	38.8 – 40.1	9.9 (± 0.12)	83.3 (± 3.9)	9.3 (± 0.32)
	Epigallocatechin gallate (EGCG)	45.3 – 46.8	9.8 (± 0.06)	4.7 (± 0.17)	23.9 (± 2.9)
	Rutin(quercetin-3-rutinoside)	52.2 – 54.5	2.9 (± 0.12)	n.d.	45.9 (± 2.56)
	Quercetin	55.3 – 55.9	n.d.	n.d.	14.7 (± 1.62)
	Total:		33.57	186.4	183.4

3.4 Effect of the extracts from S. aegyptiaca on colon cancer cell lines.

Recent studies indicate that commercial willow bark extract is capable of inhibiting the proliferation of colon cancer cells and can activate apoptotic pathways (Hostanska et al., 2007a; Vane, 1978). However, the mechanism of their function and the apoptotic pathways affected by the active elements of the extracts are still unclear. Therefore in this study, the ethanolic extracts of bark, leaves and catkins with the highest antioxidant activity and commercial salicin and aspirin were tested for their anti-proliferative and pro-apoptotic effects on colon cancer, using the human colon COX-2-positive HT-29 and COX-2-negative HCT-116 cell lines.

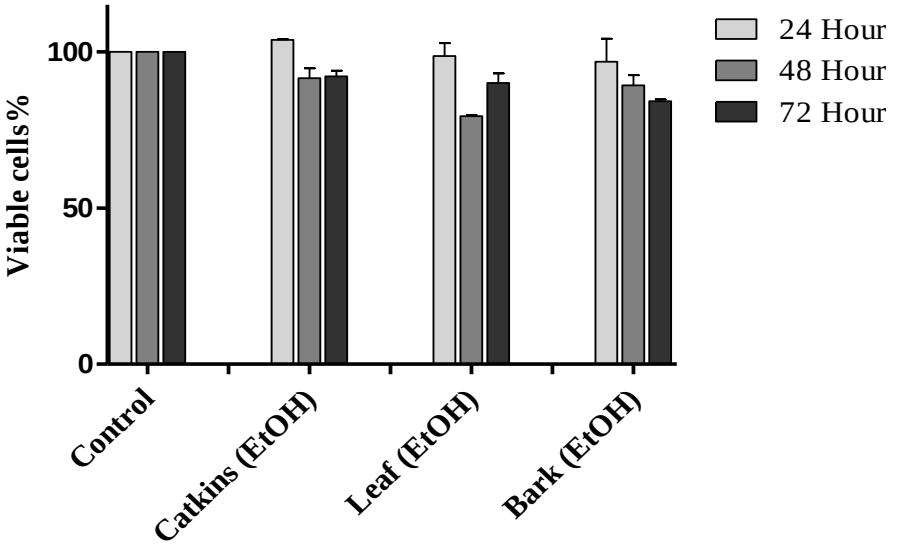
3.4.1 MTT cell proliferation assay

Growth inhibitory effect of ethanolic extracts from leaves, bark and catkins of *S.aegyptiaca* was measured by the MTT cell proliferation assay as described in Materials and Methods (Section 2.5.2). Acetyl salicylic acid (ASA) and commercial salicin were also used along with the extracts.

When the cells were treated with extracts (50 μg/ml) for 24, 48 and 72 hours to determine the anti-proliferatory effect of the extracts as a function of time, the maximum cell growth inhibitory effects of extracts for both HCT-116 and HT-29 cell lines was obtained after 48 hours treatment (Fig 12 A and B). Following this, HCT-116 and HT-29 cell lines were treated with different concentrations of extracts from 50μg/ml to 350 μg/ml for 48 hours to determine the effect of these extracts as a function of concentration. A concentration range of 100 μg/ml to 500 μg/ml of commercial salicin was used in order to achieve the concentration responsible for 50% growth inhibition in HCT-116 and HT-29 cell lines. The results are presented as the concentration of the extracts responsible for 50% cell growth inhibition (GI_{50}%) (Table 5).

The maximum cell growth inhibition in the HCT-116 cell line was observed with the ethanolic extract of bark (GI_{50}%: 202.1 ± 21 μg/ml) followed by ethanolic extracts of leaves and catkins with GI_{50}% of 272.8 ± 11.1 μg/ml and 547.7 ± 66.9 μg/ml respectively. In HT-29 cell line, ethanolic extract of bark with GI_{50}% of 363.5 ± 12.6 μg/ml exhibited the highest cell growth inhibition followed by ethanolic extract of leaves (GI_{50}%: 551.1 ± 5.5 μg/ml). The ethanolic extract of catkins showed a GI_{50}% of 1263.7 ±30.1 μg/ml in this cell line and was therefore not as efficient in inhibiting the growth of the cells. In addition, fifty percent cell growth inhibition in HCT-116 by salicin and ASA was observed at concentrations of 369.5 ± 30.3 μg/ml and 143.8 ± 3.9 μg/ml respectively. In the HT-29 cell line, fifty percent cell growth inhibition was observed with salicin and ASA concentrations of 304.2 ± 42 μg/ml and 201.1 ±12.2 μg/ml respectively. Figure 13(A and B) and Figure 14(A and B) represent the growth inhibitory percentage of all ethanolic extracts along with commercial salicin and aspirin in HCT-116 and HT-29 cell lines.

(A)



(B)

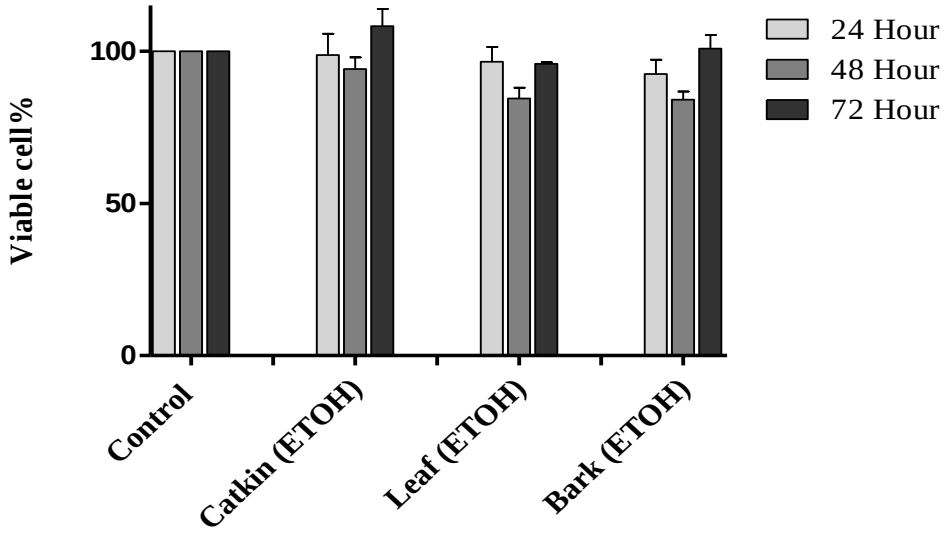
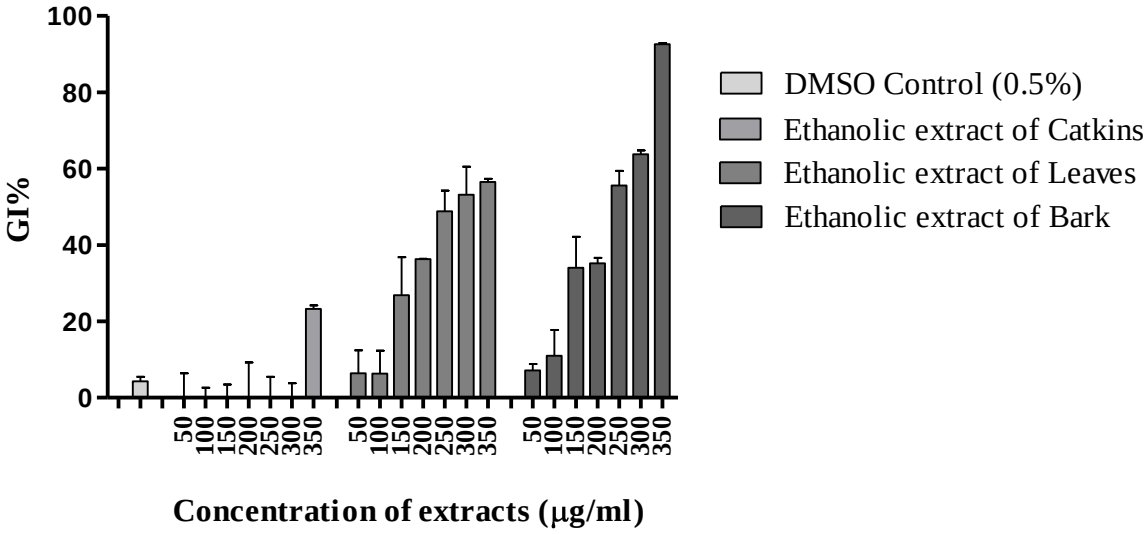


Figure 12 A and B: growth inhibitory effects of ethanolic extracts of catkins, leaves and bark of *S. aegyptiaca* (50 μg/ml) on HCT-116 colon cancer cells (A), and HT-29 colon cancer cells (B) assayed as a function of time. The name in parentheses indicates the solvent into which the parts of the plants were initially extracted.

(A)



(B)

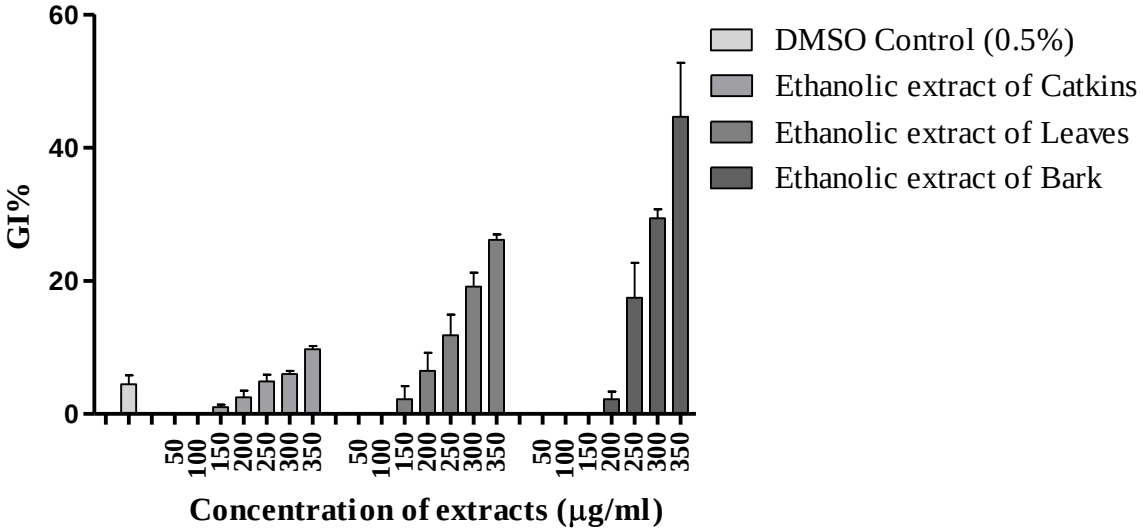
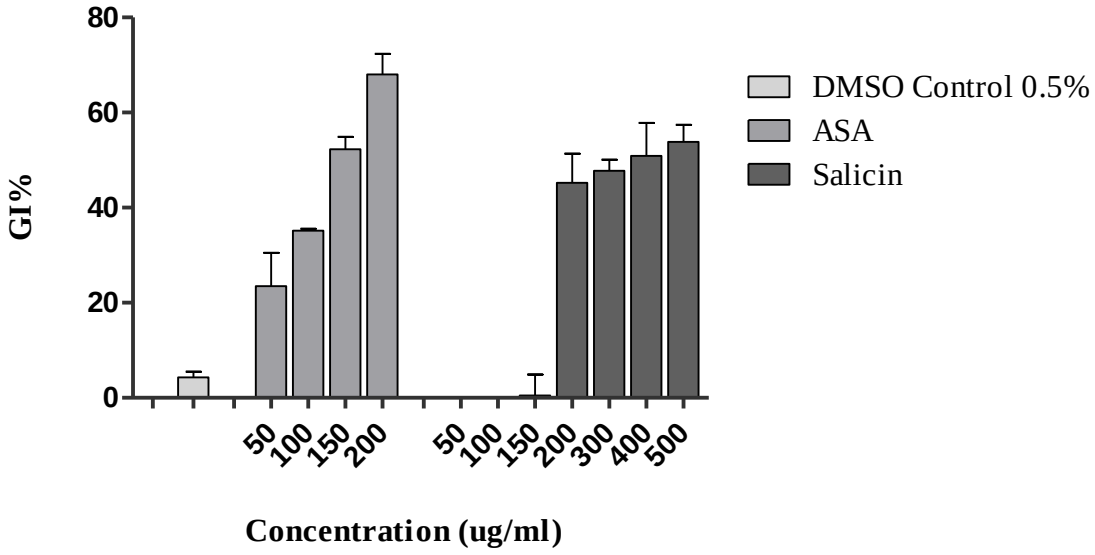


Figure 13 A and B: Growth inhibitory effects of ethanolic extracts of catkins, leaves and bark of *S. aegyptiaca* on HCT-116 colon cancer cell line (A) as a function of concentration and HT-29 colon cancer cell line (B) as a function of concentration.

(A)



(B)

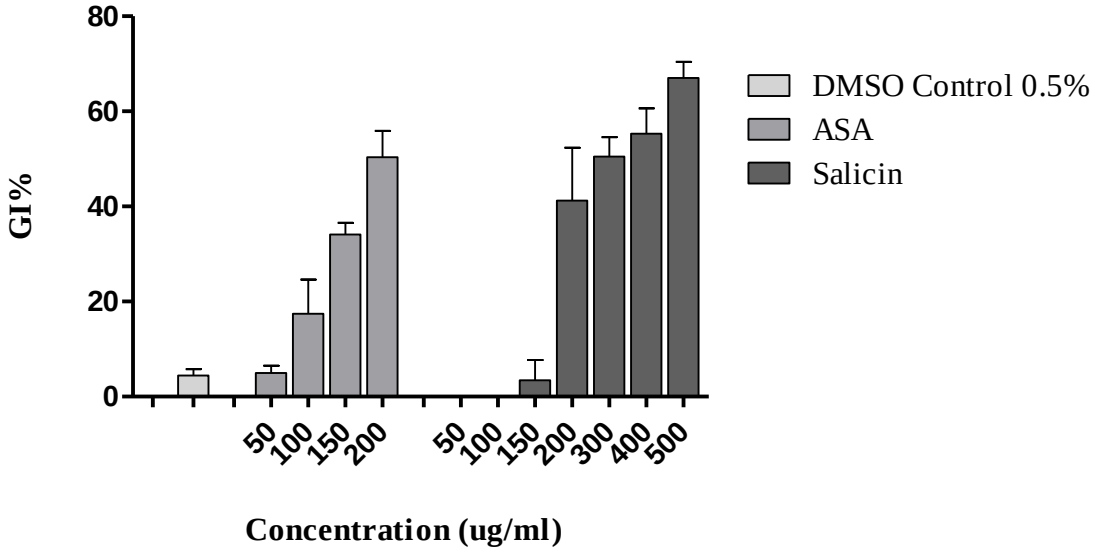


Figure 14 A and B: represent the growth inhibitory effect of acetyl salicylic acid (ASA) and commercial salicin on HCT-116 cell line (A), and HT-29 cell line assayed by MTT assay as a function of concentration after 48 hour treatment.

Table 5: Growth inhibitory potency of ethanolic extracts from catkins, leaves and bark of *Salix aegyptiaca* in HCT-116 and HT-29, represented as the concentration of

extracts responsible for 50% growth inhibition (GI_{50}). The name in parentheses indicates the solvent into which the parts of the plants were initially extracted.

Samples	GI_{50} value (μg/ml)	
	HCT-116	**HT-29**
Catkin (EtOH)	547.7 (± 66.9)	1263.7 (±30.1)
Leaves (EtOH)	272.8 (± 11.1)	551.1 (± 5.5)
Bark (EtOH)	202.1 (± 21)	363.5 (± 12.6)
Salicin	369.5 (± 30.3)	304.2 (± 42)
Acetyl salicylic acid (ASA)	143.8 (± 3.9)	201.1 (±12.2)

3.4.2 Apoptosis assays:

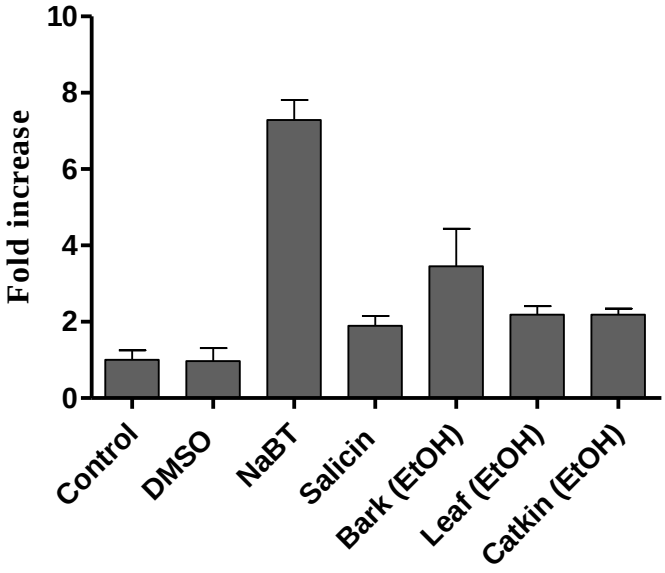
3.4.2.1 Acridine orange staining assay:

The ethanolic extracts of catkins, leaves and bark of *Salix aegyptiaca,* along with commercial salicin and aspirin were examined for their ability to induce apoptosis in HCT-116 and HT-29 cell lines. The representative microscopic images from non-apoptotic control and ethanolic extract of bark treated apoptotic cells have been showed in Figure 16 (A-C). Among the extracts, the ethanolic extract of bark with 3.5 fold increase exhibited a higher apoptotic activity, which was nearly half the activity of the positive control sodium butyrate (NaBt, 5 mM) in the HCT-116 cell line. The ethanolic extracts of catkins and leaves also induced apoptosis in HCT-116 cell line but with less potency (Fig 15 A).

In the HT-29 cell line, the ethanolic extract of bark with 1.8 fold increase could also efficiently induce apoptosis. Ethanolic extracts of catkins and leaves induced apoptosis in the HT-29 cell line with relatively similar ability. Similar results were obtained from cells treated with 250 μg/ml salicin in both HCT-116 and HT-29 cell

lines. DMSO control did not increase apoptosis in either HCT-116 or HT-29 cell lines (Fig 15 B).

(A)



(B)

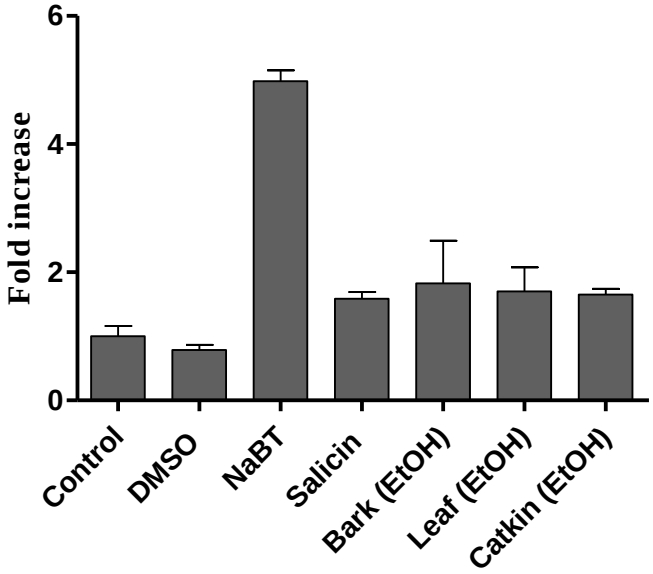
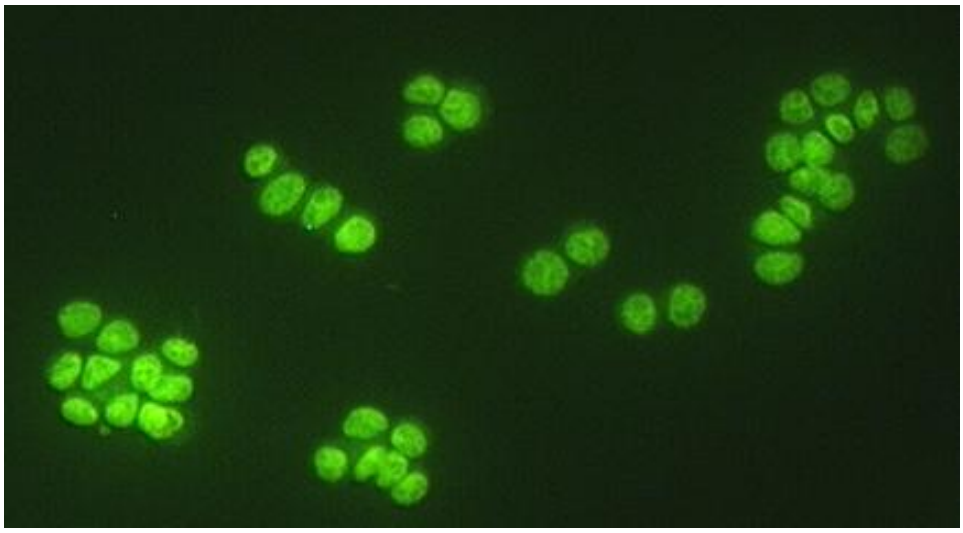


Figure 15 A: Represent the induction of apoptosis in HCT-116 cell line (A) and HT-29 cell line (B) treated with 250 μg/ml of extracts, and salicin in compare with non-treated control and sodium butyrate (NaBt, 5 mM) as positive control, determined by acridine orange staining assay.

(A)



(B)

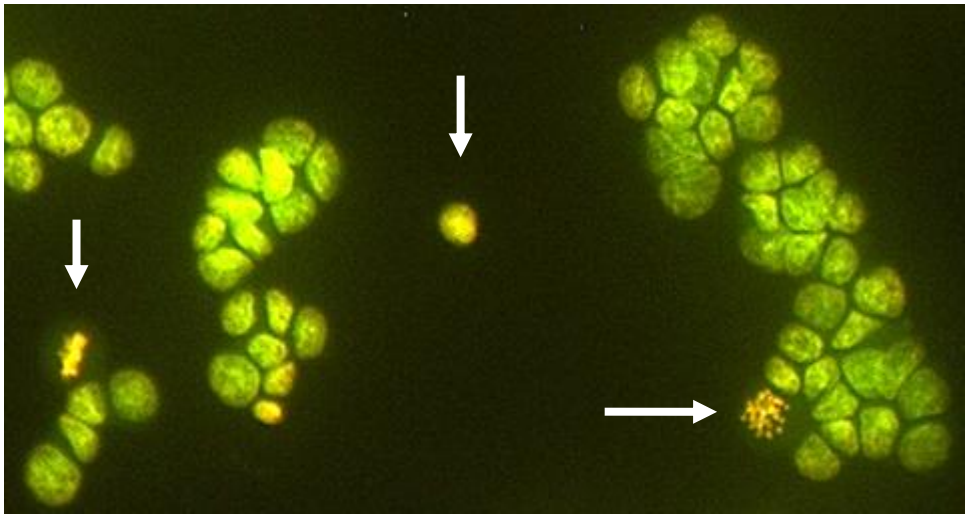


Figure 16 (A-C): representative pictures obtained from non-apoptotic HCT-116 cells (A) and apoptotic and non-apoptotic cells of HCT-116 cell line treated with ethanolic extract of bark (B) and ethanolic extract of leaves (C) assayed by acridine orange staining assay. The arrows represent the apoptotic cells.

(C)

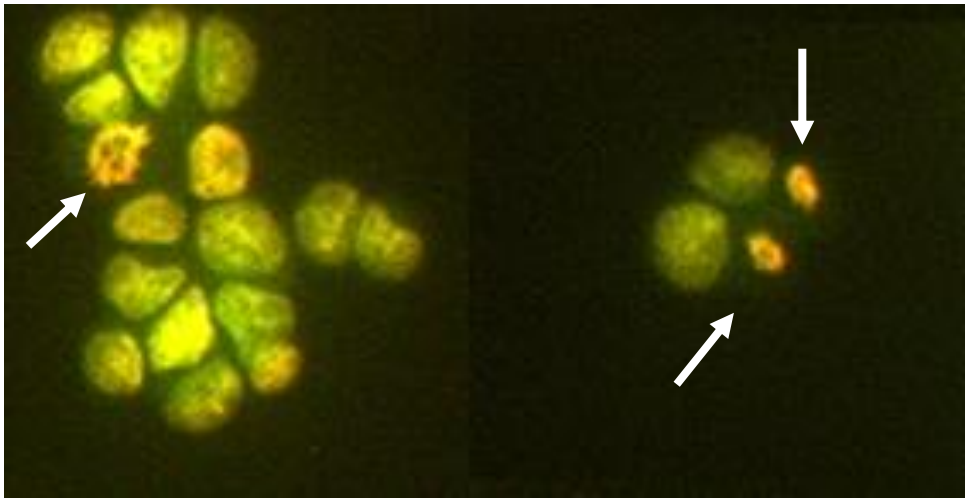


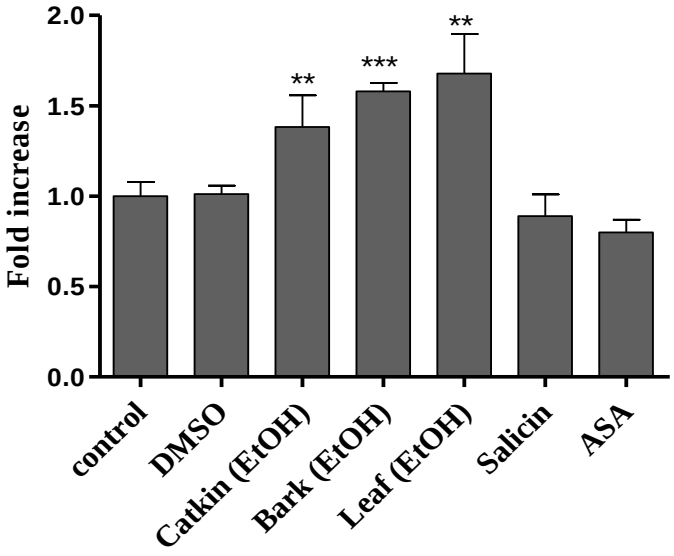
Fig. 16 Continued

3.4.2.2 Caspase 3 activity assay:

The ethanolic extracts of catkins, leaves and bark of *Salix aegyptiaca* along with commercial salicin and aspirin were also examined for the induction of apoptosis by determining their effect on caspase 3 enzyme activity in the colon cancer cell lines. The results are expressed as fold increase in caspase 3 activity compared with the non-treated control cells. The results indicate that the ethanolic extracts of leaves and bark increased caspase 3 activity 1.8 ± 0.2 and 1.6 ±0.05 folds respectively followed by ethanolic extract of catkins (1.4 ± 0.2 fold increase). All of these extracts were therefore capable of inducing apoptosis in the cell lines (Figure 17 A).

In the HT-29 cell line, incubation with the ethanolic extract of bark nearly doubled the caspase 3 activity (1.95 ± 0.04 fold) and was therefore capable of significantly inducing apoptosis. The ethanolic extracts of catkins and leaves with 1.66 ± 0.04 and 1.51 ± 0.3 fold increase respectively also showed significant apoptotic activity. Commercial salicin and aspirin didn't show any increase in caspase 3 activity in HCT-116 cell line. However, they could induce 1.46 ± 0.2 and 1.42 ± 0.21 fold increase in caspase 3 activity in the COX-2 positive HT-29 cell line respectively. The cells incubated with DMSO only had no effect on the caspase 3 activity in both cell lines (Figure 17 B).

(A)



(B)

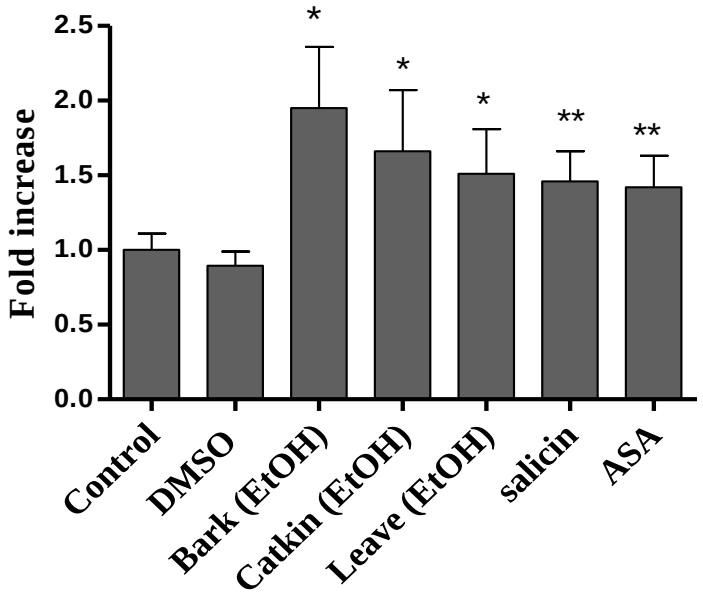


Figure 17.A and B: Represent the fold increase in caspase 3 activity in HCT-116 cell line (A) and HT-29 cell line (B) treated with 250 μg/ml of extracts, Salicin and Acetyl salicylic acid (ASA) for 24 hour in compare with non-treated and DMSO control (0.5%). Significant differences were compared with the control at *$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$ by Student's t-test.

CHAPTER 4

DISCUSSION

4.1 Discussion

Human beings have relied on a variety of plants and plant based products for medication and treatment since ancient times (Halberstein, 2005). Mixtures of phytochemicals obtained through a varied diet, as opposed to the ingestion of individual purified components, have synergistic effects that are responsible for their potent bioactive properties. This could be the reason why the health benefits observed through natural phytochemicals cannot be mimicked by a single antioxidant (Liu, 2003). Plant based antioxidants are capable of scavenging reactive oxygen species implicated in damaging cellular macromolecules and therefore can potentially protect against a wide variety of diseases such as cancer, atherosclerosis, aging etc (Olinski et al., 2007; Pan, Ghai & Ho, 2008).

Salix aegyptiaca is a member of the Salix family that has been used for centuries as a medicinal plant in many parts of the world for various health disorders. The salicin constituent of the Salix species, which is the pro-drug form of acetyl salicylic acid (aspirin), is reportedly present in high amounts in the bark, has been associated with anti nociceptive and anti inflammatory properties as well as with the induction of apoptosis in cancer cells (Chrubasik, Kunzel, Model, Conradt & Black, 2001; Fiebich & Chrubasik, 2004; Hostanska, Jurgenliemk, Nahrstedt, Abel & Saller, 2007b).

In this study we extracted leaves, bark and catkins from *S. aegyptiaca* into various solvents of increasing polarity; cyclohexane, (least polar), butanol, ethanol and water (most polar).

4.1.1 Determination of antioxidant activity

We assayed the antioxidant activities of a total of 11 different extracts from leaves, bark and catkins using the DPPH radical scavenging assay, total flavonoid content, and total phenol content. The highest antioxidant activity, as shown by the quenching of the DPPH free radical, was observed in the ethanolic extract of bark with an IC_{50} value (concentration required to quench 50% of the DPPH free radical activity) of 19 μg/ml (Figure 5 and Table 3).

This high radical scavenging activity is comparable to that of the synthetic antioxidant BHT (26 μg/ml). We also expressed the antioxidant activity (i.e. concentration capable of 50% inhibition of radical activity) in terms of quercetin equivalents, a flavonoid responsible for the potent antioxidant activity of many fruits and vegetables (Table 3). Our data indicates that the highest antioxidant activity, expressed in terms of quercetin equivalents, was found in the ethanolic extract of bark (169 ± 28 mg QE/g dried extract). The other extracts with high antioxidant activity were the butanolic extract of bark (105 ± 20 mg QE/g dried extract) and the water extract of leaves (103 ± 4 mg QE/g dried extract). Since salicin has a phenolic structure, we also examined the free radical quenching ability of purified commercial salicin by the DPPH free radical scavenging assay. However, commercial salicin as well as acetyl salicylic acid did not show any free radical quenching activity (data not shown) which indicates that the potent antioxidant capacity of the various parts of the plant bear no correlation with their salicin contents. This fact is also corroborated by the salicin content of the extracts as detected by HPLC (Table 3). When a statistical correlation analysis was conducted between the salicin content and the antioxidant activity of the extracts we could not detect any significant correlation (Spearman's r =0.4727).

4.1.2 Determination of total phenolic and total flavonoids:

We also assayed the total phenol content of the different extracts and expressed our data in terms of gallic acid equivalents (GAE). The highest total phenol content was observed in the ethanolic extract of bark (212 ± 4 mg GAE/g of dried sample) (Table 3). The phenolic content of *S. aegyptiaca* bark extracts is nearly double the total

phenolic content reported for black teas (80.5-134.9 mg/g) and green tea (65.8-106.2 mg/g) (Khokhar & Magnusdottir, 2002). The other extracts showing high total phenol contents expressed in mg GAE/g of dried sample are butanolic extract of bark (211 ± 3), water extract of leaves (163 ± 3), water extract of bark (139 ± 1), ethanolic extracts of catkin (107 ± 3), water extract of catkin (86 ± 1) and butanolic extract of catkin (81 ± 1) and ethanolic extract of leaves (64 ± 1).

The total flavonoid content of the plant was assayed for the different extracts and expressed as catechin equivalents (CE) (Table 3). The highest total flavonoid content of 479 ± 63 mg CE/g of dried extract was observed in the ethanolic extracts of bark. The other extracts with high total flavonoid content expressed as mg CE /g of dried extract are: butanolic extract of bark (419 ± 64), ethanolic extract of catkin (351 ± 2), water extract of leaves (280 ± 4), water extract of bark (243 ± 10) and ethanolic extract of leaves (165 ± 3). The total flavonoid content reported for black tea (34 mg epicatechin equivalents (ECE)/2g serving) and green tea (47 mg ECE/2g serving) (Lee, Kim, Lee & Lee, 2003 2003) all appear to be much lower than the flavonoid content of the different extracts under study here.

Statistical analyses of the antioxidant activities indicated the presence of good correlation (Spearman's r =0.938) between total flavonoid content and radical scavenging activity in quercetin equivalents at a high significance level ($p<0.0001$) (Figure 8A). A similar relationship (Spearman's r = 0.942) with high significance ($p=0.0004$) was also obtained between total phenol content and radical scavenging activity in quercetin equivalents (Figure 8B).

4.1.3 Determination of Salicin content and other phenolic compounds by HPLC

We first attempted to evaluate the salicin content in the extracts from different parts of the plant using reverse phase high performance liquid chromatography (HPLC).

Our data (Table 3) indicates that salicin could be detected in various extracts of the plant with the highest salicin content present in the ethanolic extract of bark (3.1 ± 0.04 mg/ml) (Figure 10). This was expected, since the bark of Salix species,

particularly the ethanolic extracts, are known to harbour high amounts of salicin (Guvenc et al., 2007 Dinc & Baleanu, 2007; Young, 2004).

In addition, we have shown here for the first time that water extract of catkins, which is widely marketed as a traditional health drink in parts of the Middle East, particularly in Iran, also has high salicin content at 1.8 ± 0.1 mg/ml.

The phenolic compounds gallic acid, p-coumaric acid, vanillin and caffeic acid and the flavonoids catechin, epigallocatechin gallate, rutin, quercetin and myricetin were identified and quantified in the ethanolic extract of catkins and bark and water extract of leaves by HPLC (Table 4). Among the compounds identified, catechin (83.3 ± 3.9 mg/l) and myricetin (82.1 ± 9.5 mg/l) were the most abundant polyphenols in the water extract of leaves. Myricetin (58.7 ± 4.2 mg/l) and rutin (45.9 ± 2.56 mg/l) were the predominant flavonols in the ethanolic extract of bark, and catechin (9.9 ± 0.12 mg/l) and epigallocatechin gallate (9.8 ± 0.06 mg/l) were the major flavonols identified in the ethanolic extract of catkins. Previous reports indicate a similar profile whereby the most typical flavonoids in willow bark extracted in polar solvents were the flavan-3-ols catechin, epicatechin, epigallocatechin and the catechin-3-O-(1-hydroxy-6-oxo-2-cyclohexene-1-carboxylic acid)-ester. Studies on the polyphenol content of leaves of six different Salix species have revealed that the major flavones were luteolin and apigenin and their derivatives while the major flavonols were myricetin and quercetin and their derivatives along with isorhamnetin-3-glucoside (Nyman & Julkunen-Tiitto, 2005).

Our studies have indicated that extracts with the highest antioxidant activity (such as the ethanolic extract of bark and the water extract of leaves) strongly correlate with total phenol and flavonoid content (Figure 8A and B). In addition, analysis and quantification of individual phenolic acids and flavonoids by HPLC have also indicated that these extracts are rich sources of phytochemicals, comparable to the traditionally rich sources such as tea and red wine. The myricetin content of extracts of bark (58.7 mg/l) and leaves (82.1 mg/l) of *S. aegyptiaca* were higher than those reported for red wine (7 mg/l) and black tea infusion (2-5 mg/l) (Hertog, Hollman & Vandeputte, 1993). Quercetin levels (14.7 mg/l) in the ethanolic extracts of bark

were comparable with the reported amounts in black tea infusions at 10-25 mg/l (Hertog et al., 1993). The catechin content of the water extracts of *S. aegyptiaca* leaves (83.3 mg/l) was also comparable with tea infusions (102-418 mg of total catechins/l) and red wine (27-96 mg/l) (Arts, van de Putte & Hollman, 2000).

Although willow extracts have been traditionally used as anti-inflammatory compounds for their salicin content, the presence of high amounts of phenolic compounds can contribute to the beneficial effects seen with the consumption of commercial willow extracts (Juergenliemk et al., 2007). Several reports support this hypothesis: a wide range of flavonoids such as myricetin, kaempferol, quercetin, rutin and luteolin have immunomodulatory and anti-inflammatory activities by inhibiting pro-inflammatory cytokine production and their receptors (Qin & Sun, 2005). They are also effective inhibitors of prostaglandins (Medeiros et al., 2008). The significant myricetin, rutin and catechin content of willow extracts identified in the current study could potentially contribute to the anti-inflammatory functions of willow extracts.

4.1.4 Growth inhibition activity of the extracts determined by MTT on colorectal cancer cell lines

Since the colon is directly exposed to dietary components, the consumption of phytochemicals may show considerable beneficial effects in the prevention of tumorigenesis (Bravo, 1998; Hollman et al., 1995; Lee et al., 1995).

The ethanolic extracts from catkins, leaves and bark along with commercial salicin and aspirin were examined for anti-proliferative activity on HCT-116 and HT-29 colon cancer cell lines. These extracts showed the highest antioxidant activities and the highest polyphenol contents (Table 3 and Table 4). According to the results obtained from the MTT cell viability assay (Table 5) the GI_{50}% of the ethanolic extracts of leaves and bark with the highest anti-proliferative activity were similar to the GI_{50}% of commercial salicin and ASA for both cell lines. It might explain the

ancient hypothesis about the contribution of salicin content in therapeutic effects of *Salix* species.

However, based on the salicin content of the ethanolic extract of bark as determined by HPLC (3 mg/ml), the amount of salicin available in 350μg/ml of the extract used for MTT is 105μg/ml. This amount is considerably lower than the GI_{50}% value of pure salicin (370 μg/ml for HCT116 and 304 μg/ml for HT29) to account for the growth inhibitory effects observed.

Therefore, we propose that the strong antioxidant capacity and remarkably high level of total phenolic and flavonoid contents as well as the presence of several potent polyphenols in the extracts that have known anti-carcinogenic properties could contribute to the antiproliferative effects of the extracts. Several reports about anticarcinogenic potency of phenolic and flavonoid compounds support this hypothesis. Quercetin and its derivatives can modify several signal transduction pathways associated with the development of cancer including MEK/ERK and Nrf2/keap1 and apoptotic pathways including caspases and Bcl-2 regulation (Granado-Serrano, Martin, Bravo, Goya & Ramos, 2006). Moreover, these compounds can also inhibit the transcription and activity of COX-2 (O'Leary, de Pascual-Tereasa, Needs, Bao, O'Brien & Williamson, 2004). Rodent studies indicate that dietary administration of quercetin inhibits the chemical induction of carcinogenesis, especially in the colon (Murakami, Ashida & Terao, 2008). According to our data quercetin and quercetin rutinoside (rutin) are present in significant amounts in ethanolic extract of bark which shows strong anti-proliferative activity in both HCT-116 and HT-29 cell lines.

Catechin, epigallocatechin gallate and related polyphenols are other flavonoids that have been reported to show antiproliferative and pro-apoptotic effects in stomach and colon cancer cell lines. ATF3, a transcription factor with proapoptotic activity in HCT-116 cell lines is activated by epicatechin gallate (Cho, Sukhthankar, Lee, Yoon & Baek, 2007). In addition, epigallocatechin gallate (EGCG) can inhibit matrix metalloproteinases (MMPs) associated with tumor invasion and metastasis (Isemura,

Saeki, Kimura, Hayakawa, Minami & Sazuka, 2000). Both catechin and epigallocatechin gallate have been found in significant amounts in the extracts of leaves and bark of *S. aegyptiaca* respectively (Table 4) and could contribute to the observed anti-proliferative effects in the colon cancer cell lines.

4.1.5 Apoptotic activity of extracts determined by acridine orange staining assay and caspase 3 activity assay

The apoptotic activity of ethanolic extracts and commercial salicin and aspirin, analyzed by acridine orange staining assay, indicated that the ethanolic extract of bark is capable of inducing a 3.5 fold increase in apoptosis over control HCT-116 and 1.8 fold increase over control HT-29 cell lines. The apoptotic effects of the extracts were also assayed by caspase 3 activity assay. The results indicate at 250μg/ml the ethanolic extracts of leaves and bark show a 2-fold increase in caspase 3 activity and are therefore capable of inducing apoptosis in HCT-116 and HT-29 cell lines. Interestingly, commercial salicin and aspirin (which can inhibit COX-2) did not increase the caspase 3 activity in the COX-2 negative HCT-116 cell line. However, both compounds could increase the caspase 3 activity in the COX-2 positive HT-29 cell line which could indicate the COX-2 inhibitory and a consequent pro-apoptotic function of these compounds on this cell line.

CHAPTER 5

CONCLUSION

5.1 Conclusion

This work was the first to characterize extracts from bark, leaves and catkins of Salix *aegyptiaca* in terms of antioxidant content, polyphenol content (total and individual) as well as the growth inhibitory effects of a selected group of these extracts on colon cancer cell lines. This species of plant is found only in the Middle East and its essence from the flowers is consumed as a traditional health promoting drink in Iran. Our detailed analyses have shown that some of these extracts have very high antioxidant contents and are rich in salicin, which is known for its anti-inflammatory activity. When a selected group of extracts were analyzed for their effects on colon cancer cell lines, they showed significant growth inhibitory properties as demonstrated by the MTT assay. The extracts could also induce apoptosis in the two colon cancer cell lines as shown by acridine orange staining and caspase 3 activity assays.

We believe that this work has highlighted the importance of traditional herbal formulations on diseases of modern times. Future research will indicate whether these phytochemical rich extracts have any effects on the different signaling pathways that are associated with the development of cancer. These studies will further delineate the importance of the consumption of a combination of antioxidants in the fight against cancer.

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APPENDIX A

HPLC CHROMATOGRAMS

A.1 Results of HPLC determination of salicin in the extracts of Salix aegyptiaca

(B)

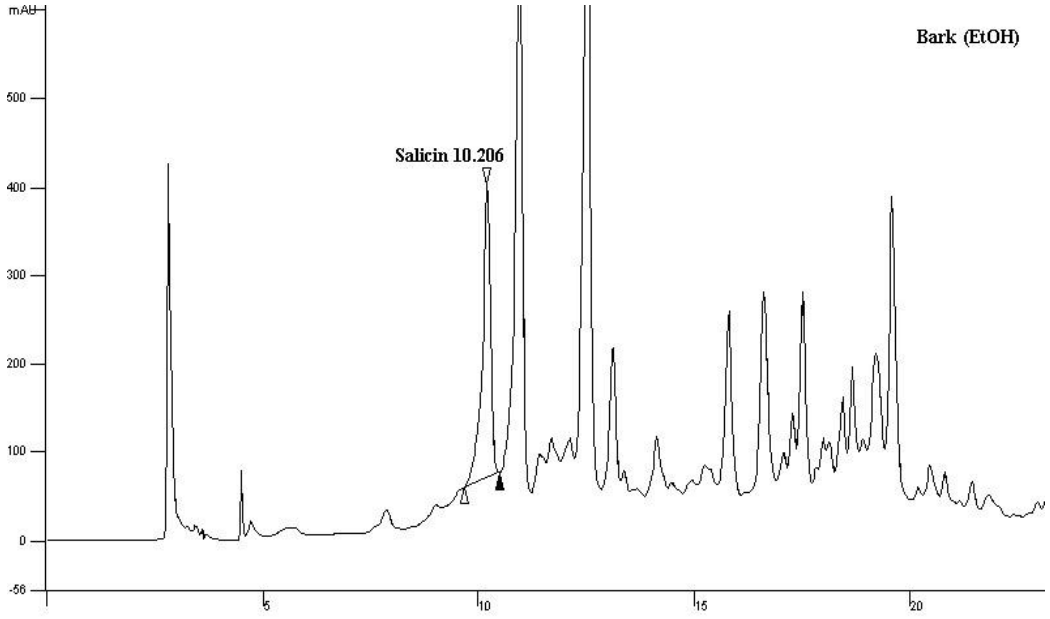
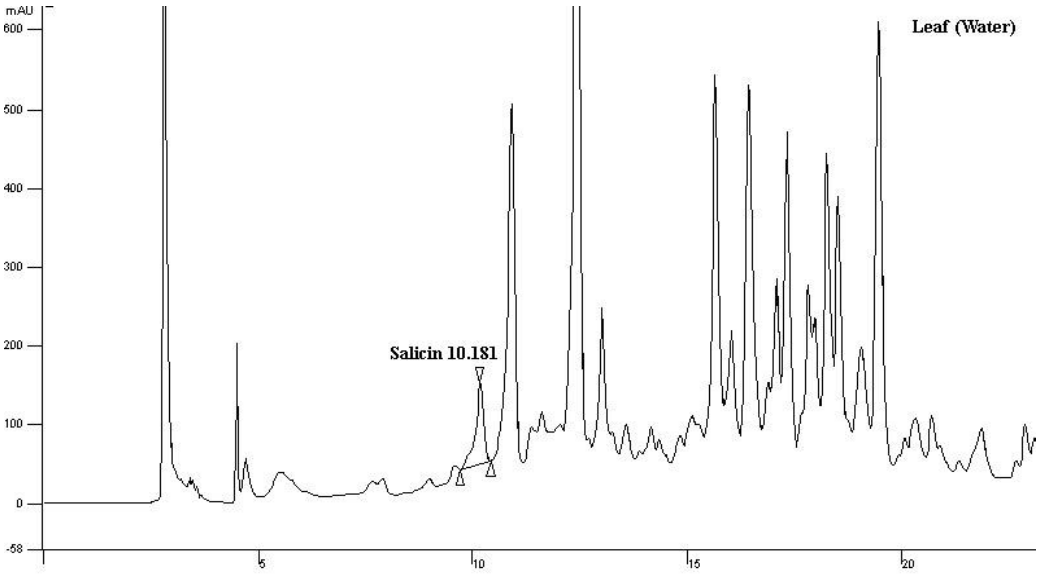


Fig. 9 B-L. Chromatogram of the standard ethanolic extract of bark (B), water extract of leaf (C), butanolic extract of bark (D), water extract of bark (E), ethanolic extract of leaf (F), ethanolic extract of catkin (G), butanolic extract of catkin (H), butanolic extract of leaf (I), water extract of catkin (J), cyclohexane extract of leaf (K) and cyclohexane extract of bark (L). HPLC was carried out with an Odosil column with a mobile phase 3% to 48% of acetonitrile in a mixture of water/ trifluoroacetic acid (TFA) (100:0.05 v/v), flow rate of 1ml/min. The column temperature was maintained at 30°C, and detection of salicin was carried out at 268 nm. Identical conditions were used for the standard salicin and all of the extracts.

(C)



(D)

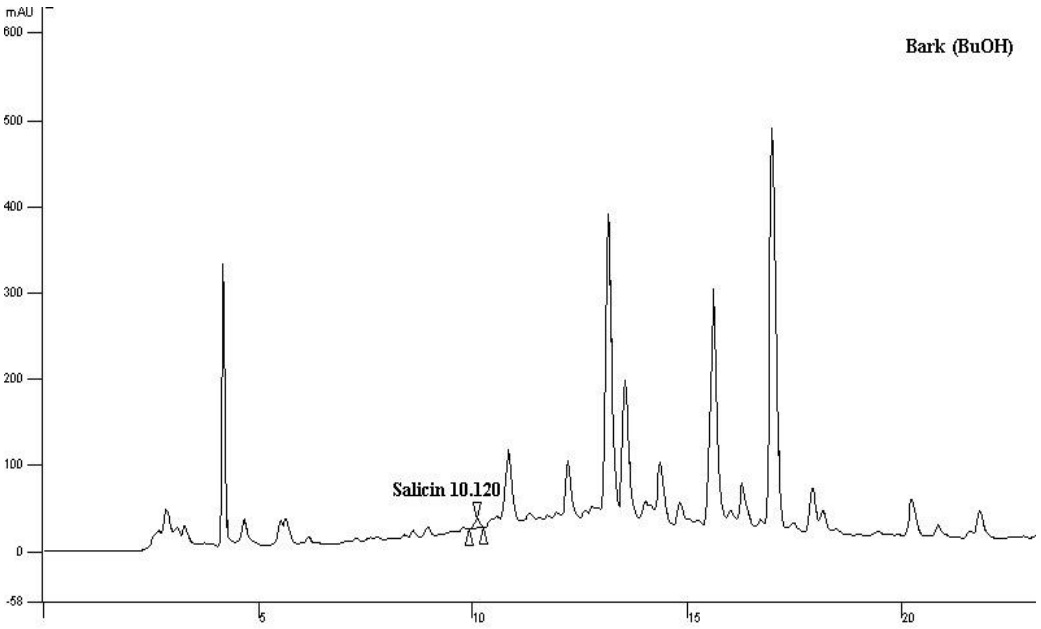
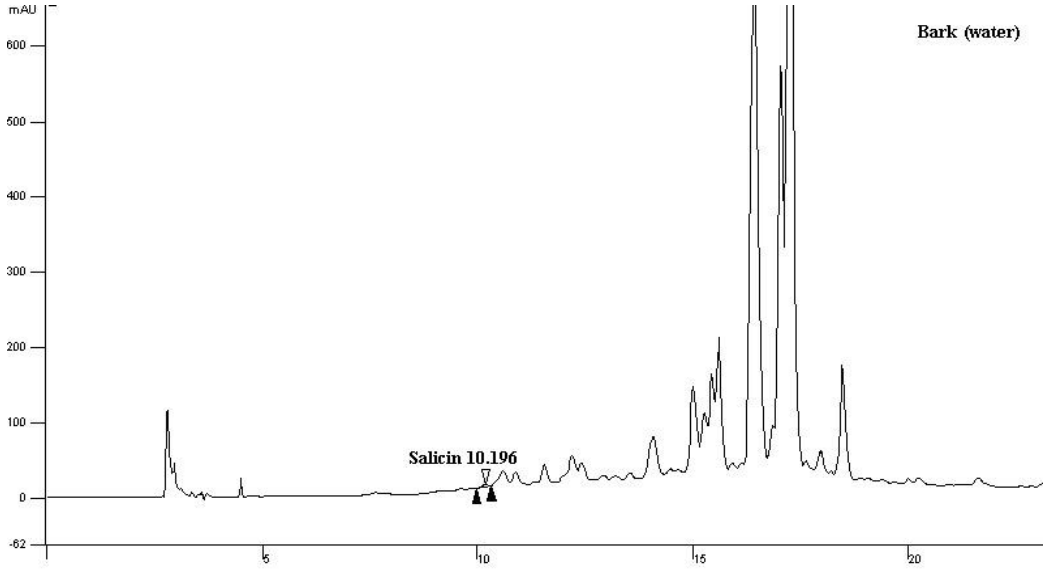


Fig. 9 Continued

(E)



(F)

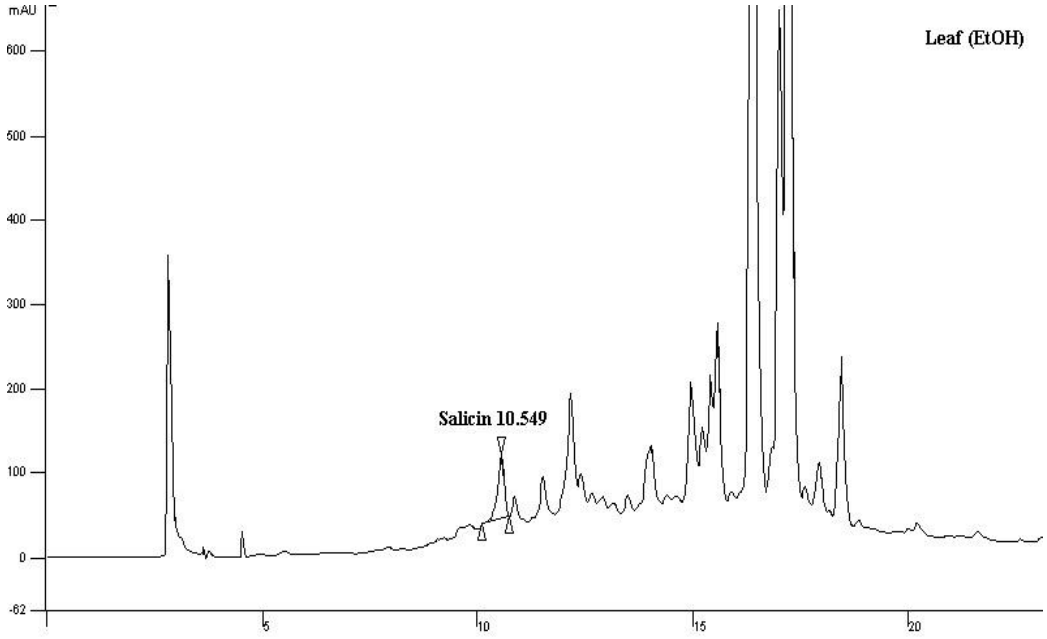
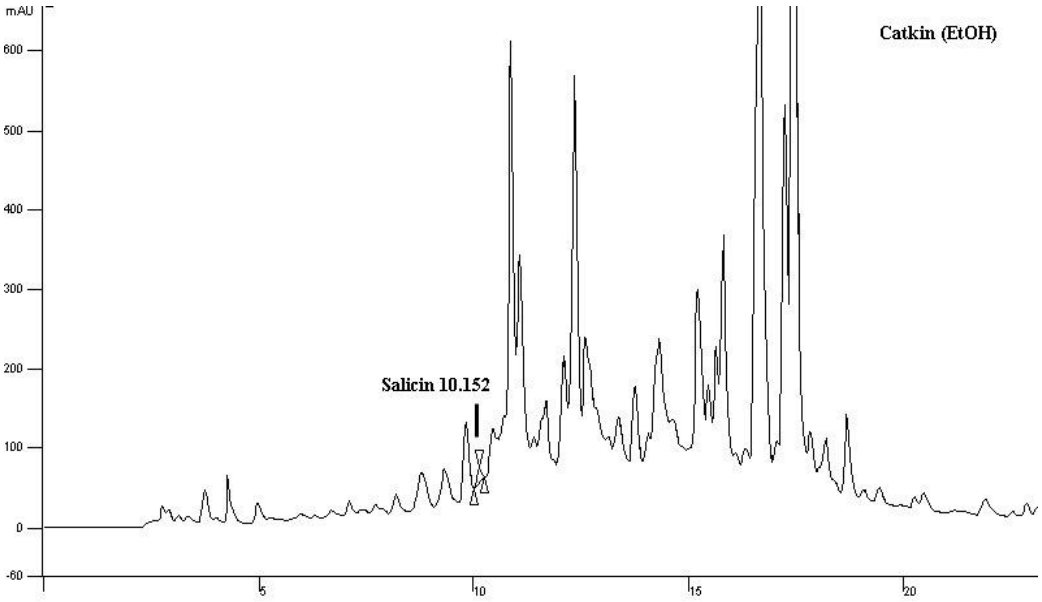


Fig. 9 Continued

(G)



(H)

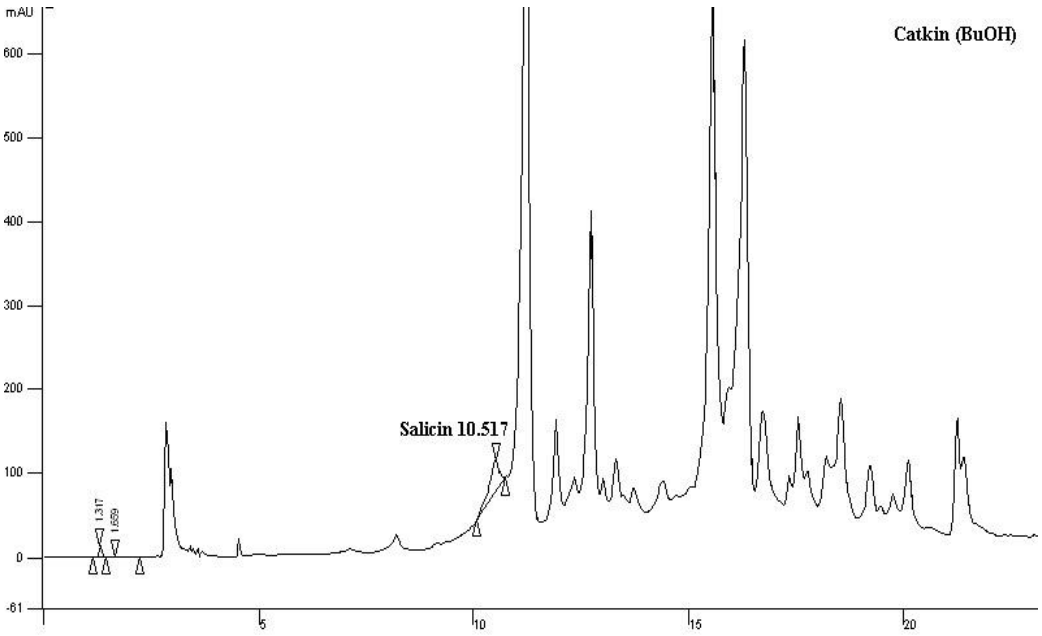
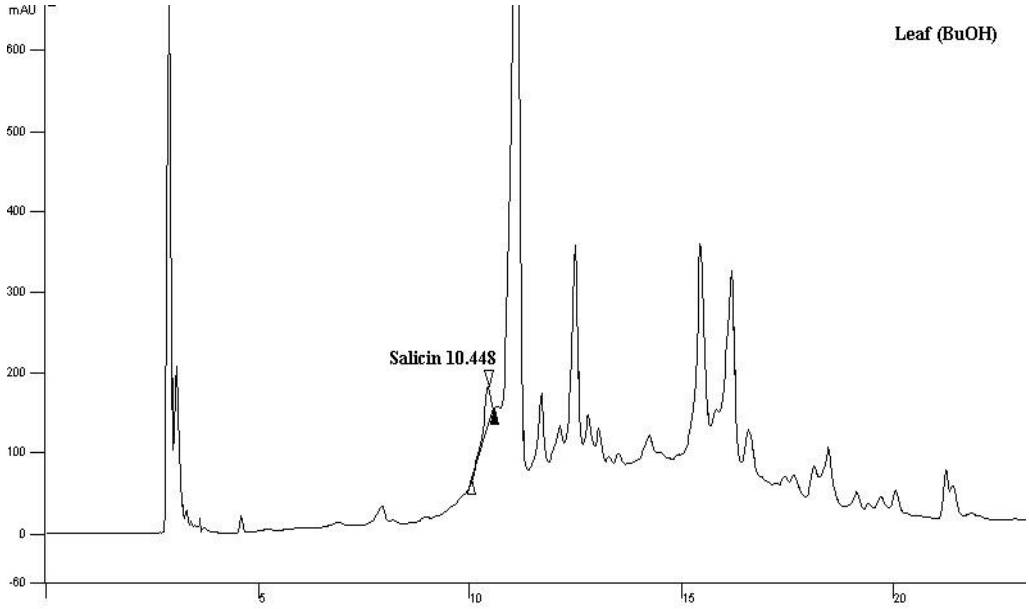


Fig. 9 Continued

(I)



(J)

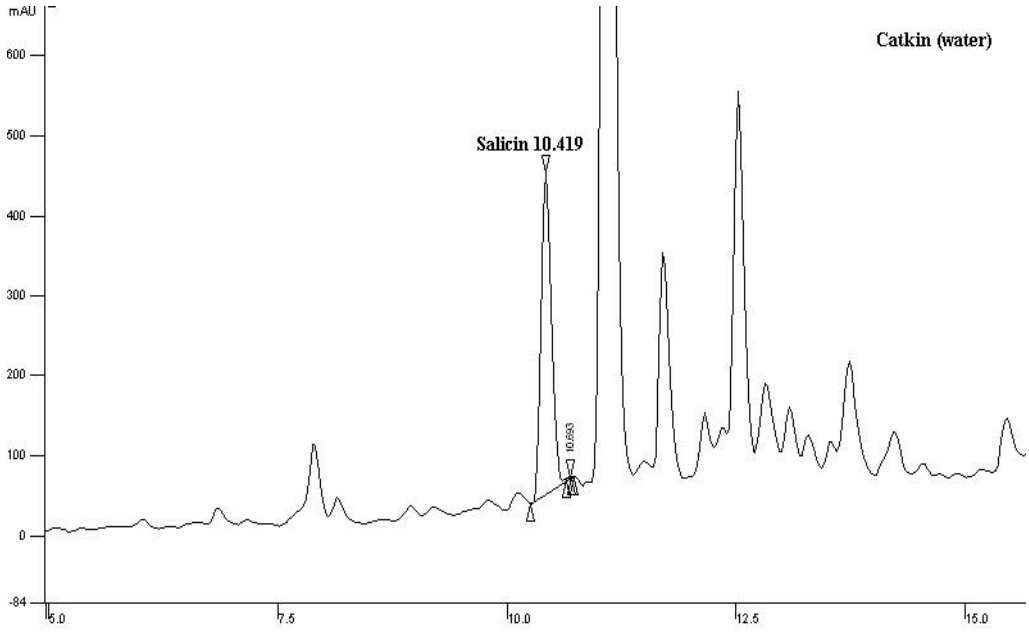
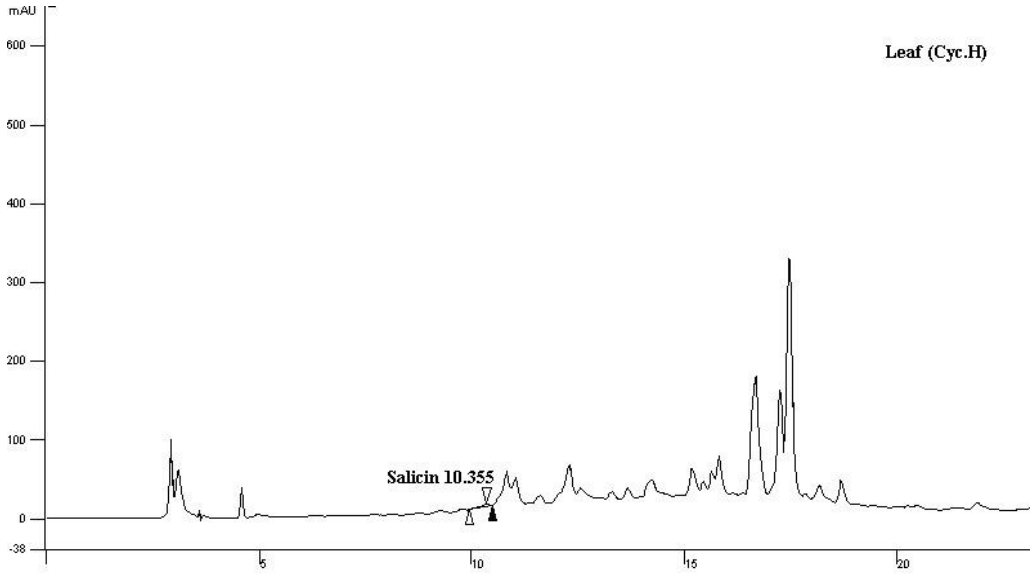


Fig. 9 Continued

(K)



(L)

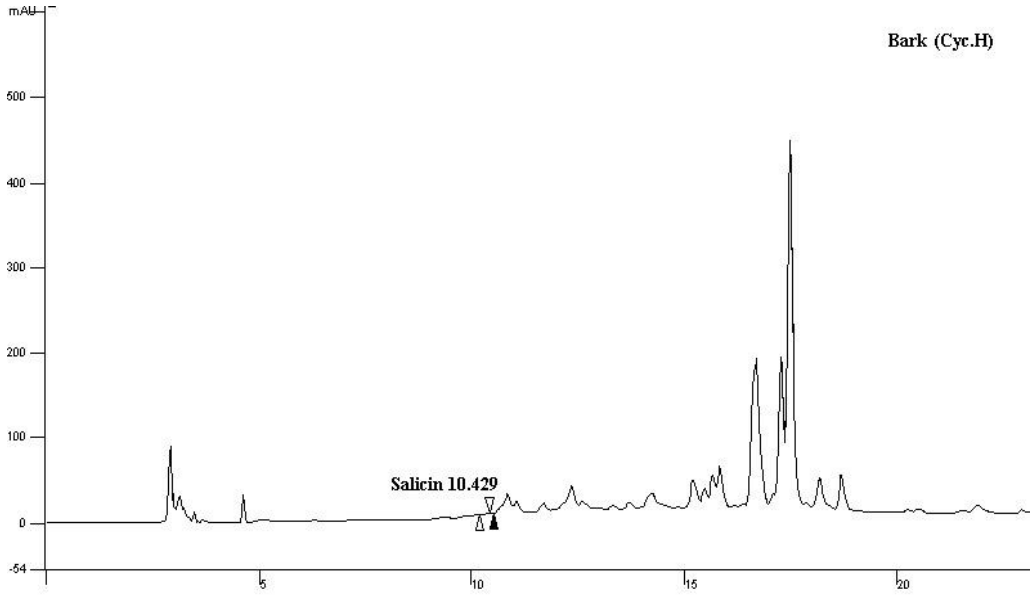


Fig. 9 Continued

A.2 Results of HPLC determination of phenolic and flavonoid compounds in the extracts of Salix aegyptiaca

(D)

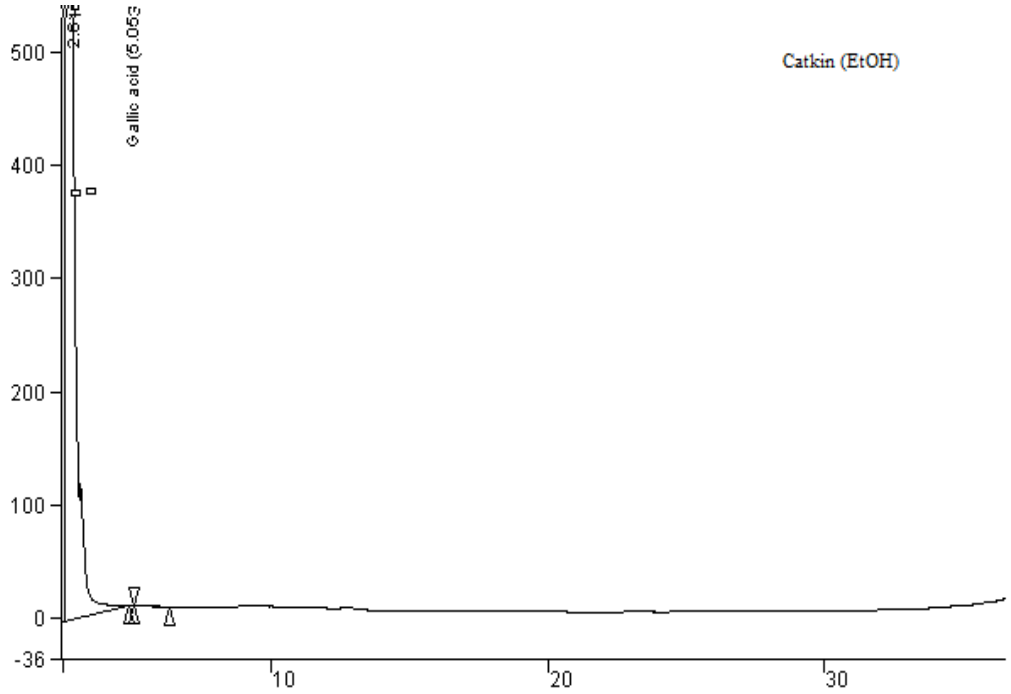
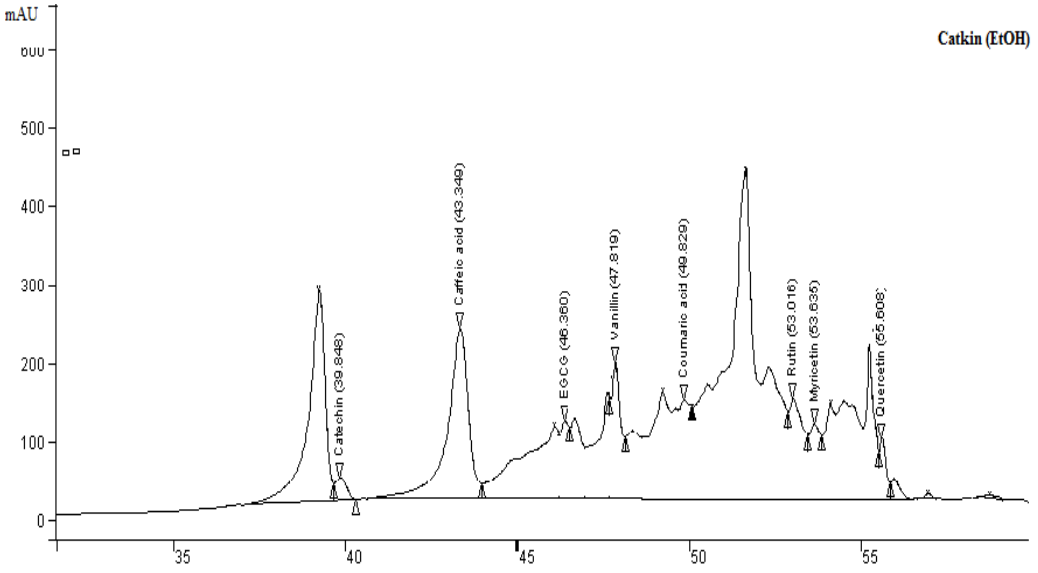


Fig 11(D-O): Chromatograms of gallic acid observed at 280 nm in the ethanolic extract of catkin (D), catechin, EGCG, vanillin detected at 280 nm in the ethanolic extract of catkins (E), caffeic acid and p-coumaric acid detected at 320 nm in the ethanolic extract of catkin (F), myricetin, rutin and quercetin detected at 360 nm in ethanolic extract of catkin (G), gallic acid observed at 280 nm in the ethenolic extract of leaves (H), catechin, EGCG, vanillin detected at 280 nm in the ethanolic extract of leaves (I) caffeic acid p-coumaric acid detected at 320 nm in the ethanolic extract of leaves (J), I: myricetin, rutin and quercetn detected at 360 nm in the ethanolic extract of leaves (K), gallic acid observed at 280 nm in the ethanolic extract of bark (L) catechin, EGCG, vanillin detected at 280 nm in the ethanolic extract of bark (M), caffeic acid and p-coumaric acid observed at 320 nm in the ethanolic extract of bark (N), myricetin, rutin and quercetin detected at 360 nm in the ethanolic extract of bark (O).

(E)



(F)

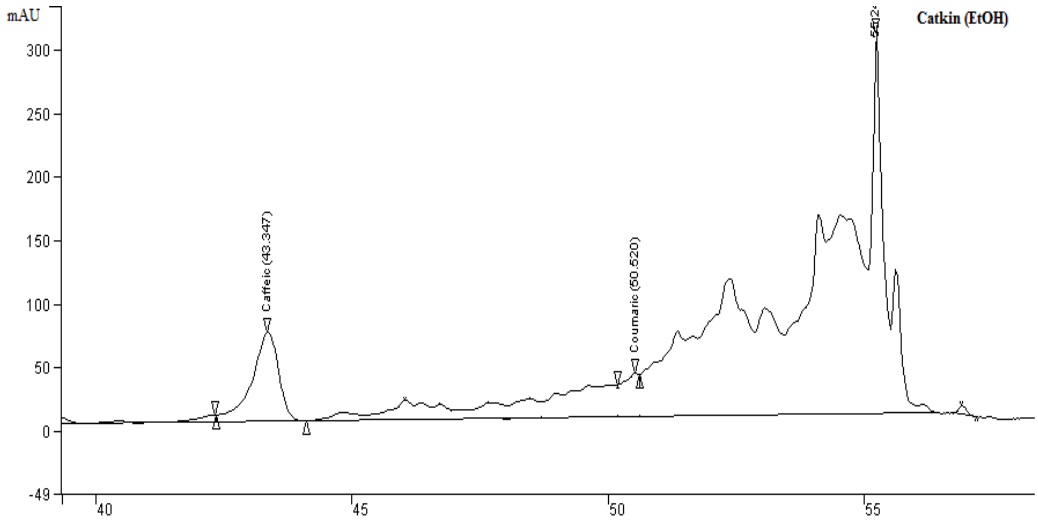
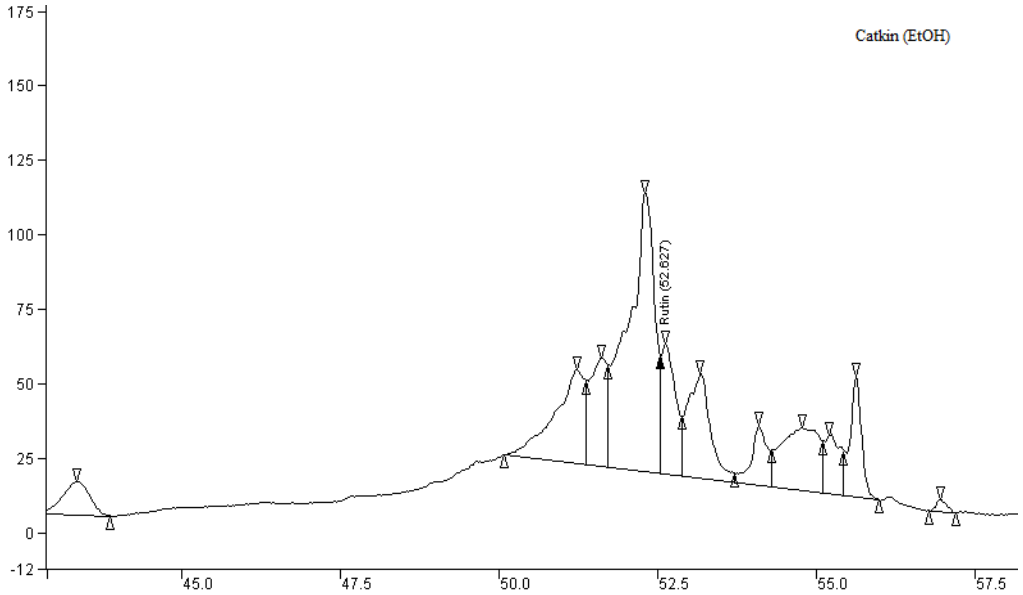


Fig 11 Continued

(G)



(H)

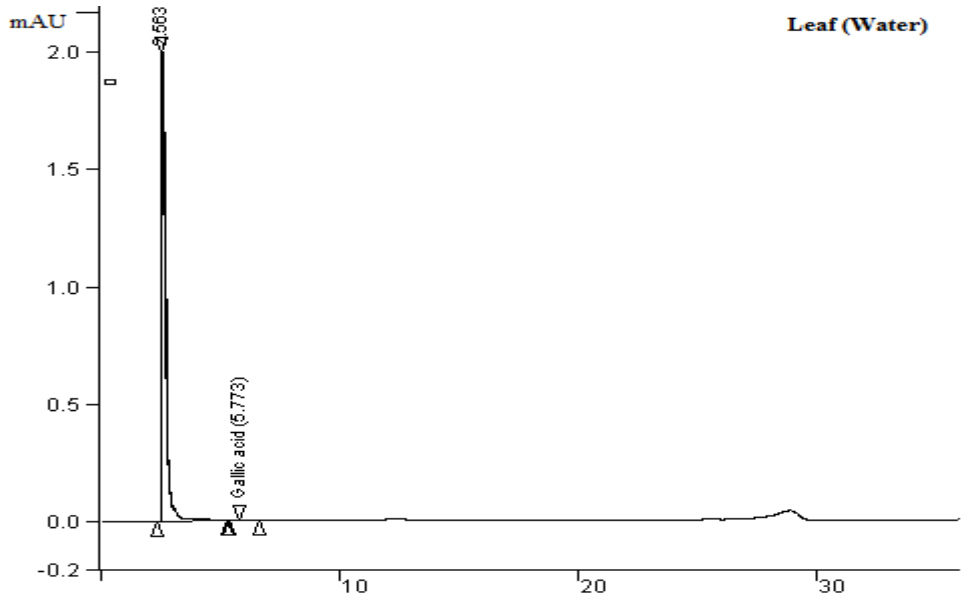
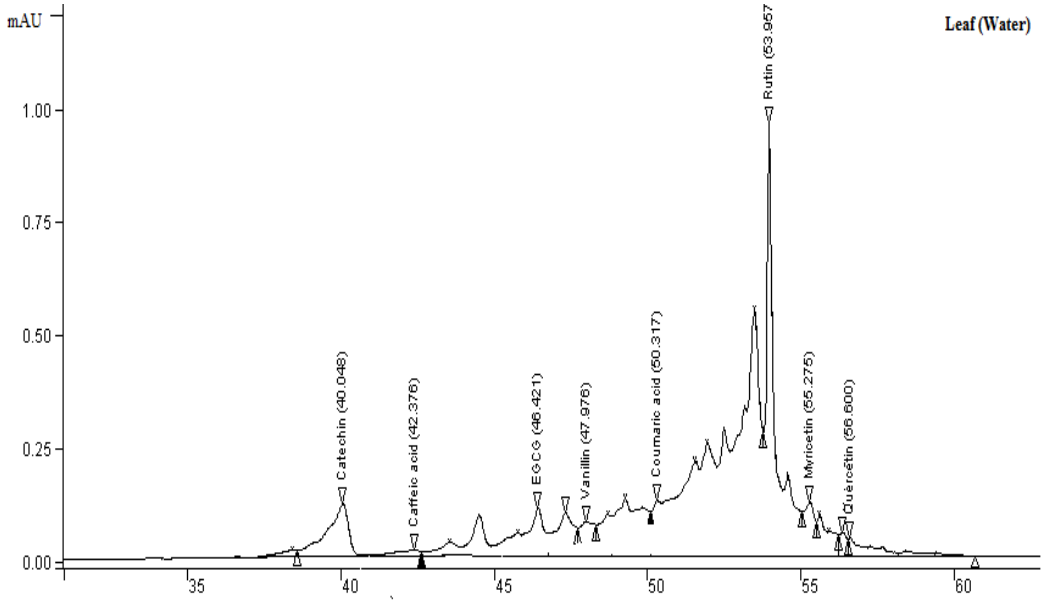


Fig 11 Continued

(I)



(J)

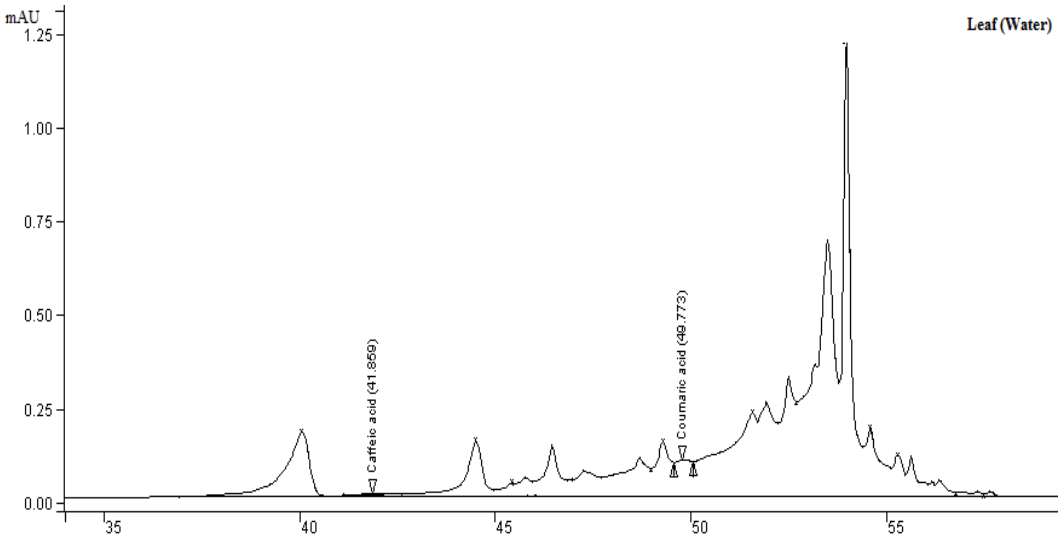
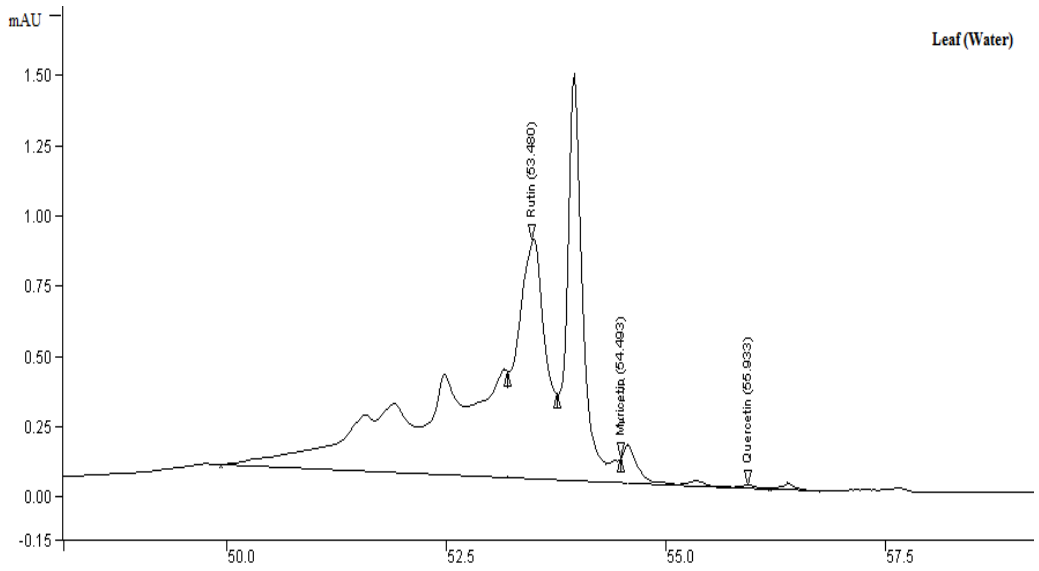


Fig 11 Continued

(K)



(L)

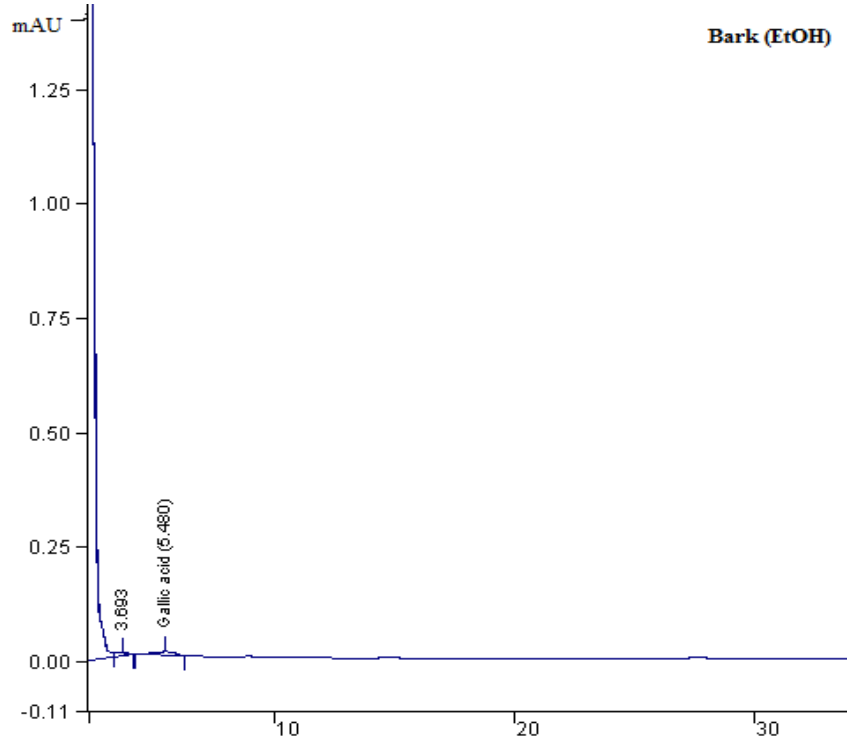
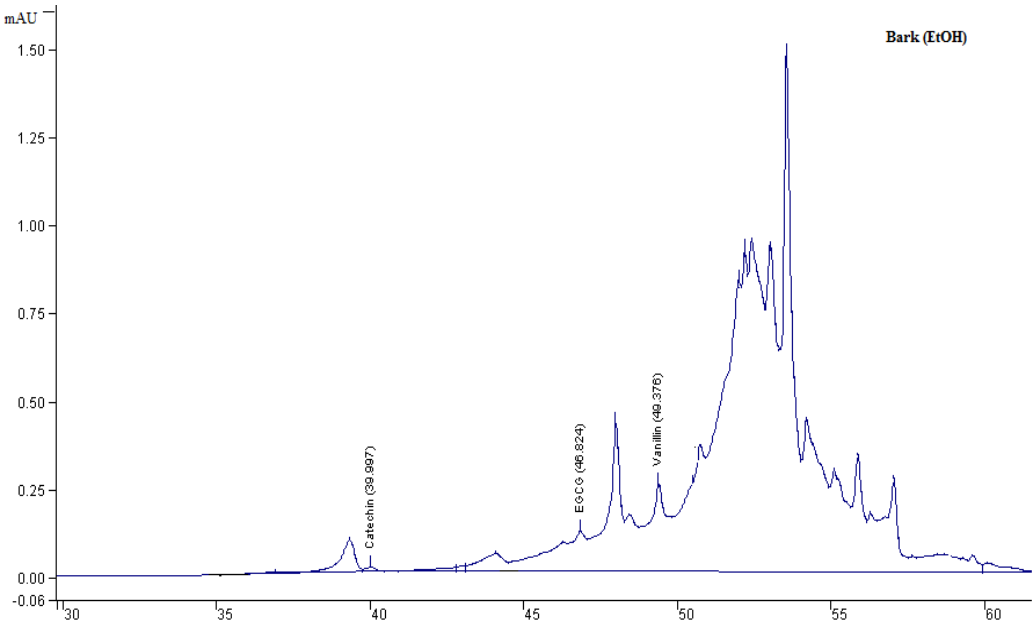


Fig 11 Continued

(M)



(N)

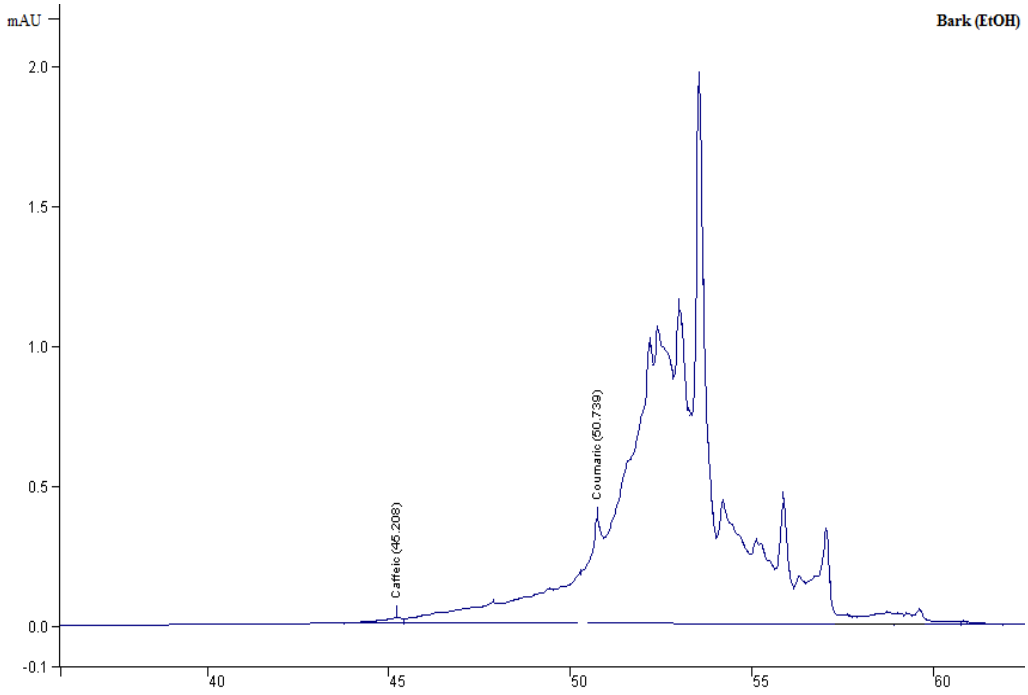


Fig 11 Continued

(O)

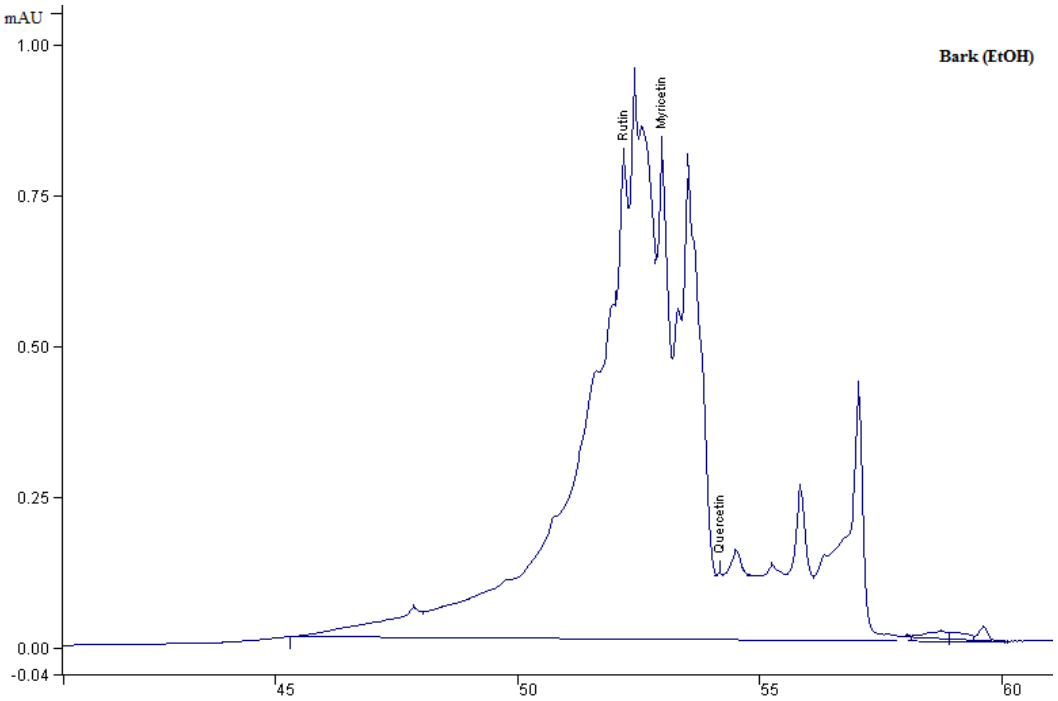


Fig 11 Continued

APPENDIX B

THE EQUATIONS OF GROWTH INHIBITORY PERCENTAGE

Table B.1: The equations of GI% of ethanolic extracts of *S. Aegyptiaca*, Salicin and Aspirin obtained from MTT cell viability assay in HCT-116 and HT-29 colon cell lines.

Samples	**Equations (HCT-116)**	**Equations (HT-29)**
Catkins (EtOH)	y = 0.1385x - 25.855 R^2 = 0.9938	y = 0.0443x - 5.9815 R^2 = 0.992
Leaves (EtOH)	y = 0.1827x + 0.1518 R^2 = 0.9703	y = 0.1212x - 16.787 R^2 = 0.9942
Bark (EtOH)	y = 0.2859x - 7.8124 R^2 = 0.9996	y = 0.3158x - 64.788 R^2 = 0.9944
Salicin	y = 0.0289x + 39.323 R^2 = 0.9984	y = 0.0834x + 24.627 R^2 = 0.9909
ASA	y = 0.3213x + 3.7843 R^2= 0.9981	y = 0.3059x - 11.528 R^2 = 0.996

APPENDIX C

DETERMINATION OF RADICAL SCVENGING EFFECT (IC_{50}%) OF ETHANOLIC EXTRACT OF BARK FROM S. AEGYPTIACA

Table C.1: Represent the decrease in the absorbance of DPPH methanolic solution quenched by different concentration of the ethanolic extract of bark of S. aegyptiaca and the percentage of inhibition in radical activity related to each concentration.

Concentration μg/ml Bark (EtOH)	**Absorbance (Average) (nm)**	**DPPH Scavenging Effect (%)**
0	0.8006	0
5	0.680333	15.02
10	0.557	30.43
15	0.534	33.3
20	0.420667	47.46
25	0.314	60.78
30	0.226	71.77
35	0.153333	80.85

The percentage of DPPH free radical quenching activity was determined from the formula:

$$\text{DPPH Scavenging Effect (\%)} = \left(\frac{A_{DPPH} - A_{Extract}}{A_{DPPH}}\right) \times 100$$

(A)

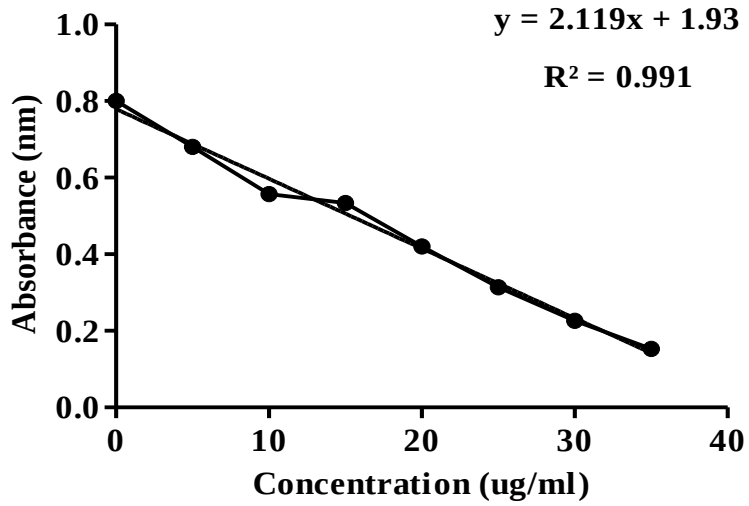


Fig. 12 A: Linear regression in the absorbance of DPPH methanolic solution quenched by different concentration of the ethanolic extract of bark of S. aegyptiaca.

(B)

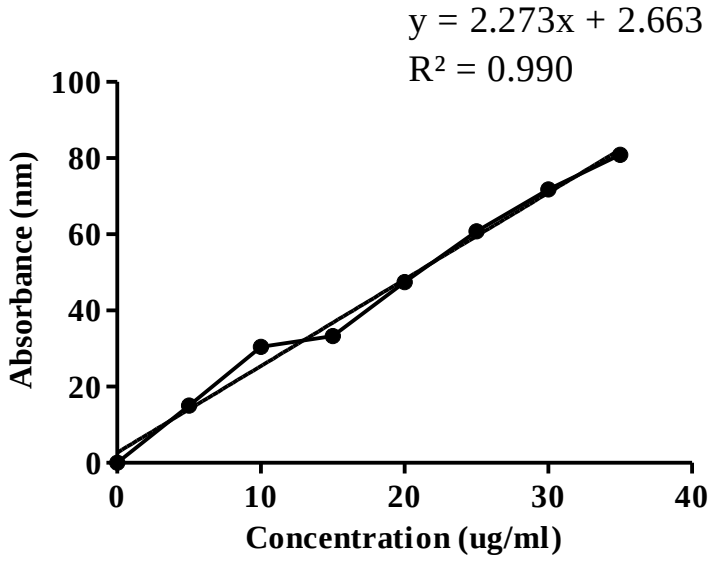


Fig. 12. B: DPPH scavenging effect (%) induced by different concentration of the ethanolic extract of bark of S. aegyptiaca at time point of 1 hour using the formula. The equation generated from this chart was used to determine the concentration of each extract required to quench 50% of the DPPH free radical activity (IC_{50} value).

The concentration of the extract required for 50% DPPH scavenging effect (X) was determined as followed:

$y = 2.273x + 2.663$

$Y = 50$ (Indicating 50% scavenging effect)

$X = (50 – 2.663) / 2.273$

$X = 20.83$ μg/ml

IC_{50}% of 19 μg/ml was obtained from the mean of repeated experiments.