Potent Anti-acne Activity and Biological Constituents of Terminalia laxiflora and Acacia nilotica as Sudanese Medicinal Plants

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（スーダン産薬用植物 *Terminalia laxiflora* と *Acacia nilotica* の強力な抗アクネ活性と活性成分に関する研究）

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The United Graduate School of Agricultural Science,

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Science of Biological Resources

(Gifu University)

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Summary

Acne vulgaris is the most common skin disease in the world and is not a simple disease, which may sometimes lead to psychosomatic influence such as depression or frustration. Many acne patients undergo complete resolution of their lesions without any remaining symptoms, whereas other patients have continuous acne or long-term consequences, such as scarring and keloids. The bacterium *Propionibacterium acnes* play a central role in the current concept of acne pathogenesis. Number of antibiotics resistant to acne-inducing bacterial strains has been increasing in the past years. Natural substances from plants are become promising candidates to treat this disease. On the other hand plenty of free fatty acids was detected in acne lesions forms as a result by the effect of *P. acnes* lipase on sebaceous triglycerides. *P. acnes* lipase itself can act as a chemotactic factor; in addition these free fatty acids lead to induces serious inflammation.

Oxidant/antioxidant imbalance leads to increased production of free radicals that cause many diseases. The skin is constantly exposed to oxidative stress induced by reactive oxygen species (ROS), that are generated both from endogenous sources, such as enzyme activity or activated neutrophils, and external pro-oxidant stimuli, such as ultraviolet radiation (UV). Over production of ROS produced by *P. acnes* and neutrophils are involved in the irritation and destruction of the follicular wall, responsible for the inflammatory progression of acne. Thus antioxidant oral supplementation or topical application may be an effective approach in improving the efficacy or avoiding the potentially deleterious effects of the therapeutical agents.

Herbal remedies, including those for skin disorders, are currently gaining popularity among patients and to a lesser degree among physicians. Traditional medicine is widely

used as source of primary healthcare for a majority of people in Sudan. The anti-acne activity of Sudanese medicinal plants specifically and African medicinal plants as general against acne causing factors have been rarely addressed, even though attention has been given to this disease in other part of world.

The present study was undertaken with the primary objective of finding Sudanese medicinal plants that have anti-acne potency based on antibacterial, lipase inhibition and antioxidant activity. Total phenolic, flavanoids and tannin content of active extracts were also determined. A second objective was to determine the active compound for anti-acne activity from the potent Sudanese medicinal plants. I initiated this work with a five weeks expedition to Sudan in March 2010. Forty plant species were collected from mainly two locations in the districts of Al Qadarif and Khartoum. During the expedition plant has been identification and authentication by the staff of Khartoum University faculty of agriculture and faculty of forest.

This thesis is divided into four chapters. Chapter one is general introduction, research objectives and thesis outline. Chapter two is the screening of anti-acne potency of selected Sudanese medicinal plants and chemical analysis of active extracts. The chapter three is anti-acne activity of tannin related compounds isolated from *Terminalia laxiflora* Engl & Diels and last chapter explore the potency of *Acacia nilotica* pods as anti-acne agent and general conclusion

In practical parts of this thesis chapter two, I have screened 104 crude extracts belonging to 40 plant species distributed among 28 families. The methanol and 50% ethanol (v/v) extracts were tested *in vitro* for their potential anti-acne activity. The activities of these extracts were determined using an antibacterial assay against

Propionibacterium acnes, a lipase inhibitory assay, and l,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay.

Among the extracts used in this study the species belong to Combretaceae family comparatively showed a good antibacterial activity, specifically methanol and 50% ethanol extracts from *T. laxiflora* wood showed the best antibacterial activity against *P. acnes* (MIC-minimum inhibitory concentration=130 µg/ml), followed by methanol extract of *Terminalia brownii* (Bark) and all of *Combretum hartmannianum* (wood) extracts exhibited a medium antibacterial activity (MIC 500 µg/ml).The 50% ethanol extracts of *Abrus precatorius* L. seed , *T. laxiflora* wood and methanol extract of *Acacia nilotica* (L) pods showed lipase inhibitory activity more than 70% at 500 µg/ml. The methanol extracts of *A. nilotica* (L) pods showed the outstanding DPPH radical scavenging activity (IC_{50} 1.32 µg/ml) even that the (+) catechin as positive control. Total phenolic, flavanoid and total tannin contents of selected plant extracts shown anti-acne activities were investigated as well. Almost all selected extracts contained phenolic compound. The highest level of flavanoids (38.87 µg/mg) were detected in *T. brownii* bark whereas the highest amount of tannin were detected in *A. nilotica* (L) bark (88.01%). The chemical analysis clarify that hydrolysable tannins may included in *T. laxiflora* wood extracts while condensed tannin would be included in *A. nilotica* (L.) and *T. brownii* barks.

In chapter three, we are focused on *T. laxiflora* methanolic wood extract, which has been selected during previous screening experiments for anti-acne agents. The extract was separated by medium pressure liquid chromatography (MPLC) using ODS column and LH-20 gel column chromatography. Methanol, water in the different ratio and 70%

acetone was using as eluting solvent. Based on the biological assay guided fractionation; four tannin related compounds were isolated as ellagic acid, flavogallonic acid dilactone, terchebulin and gallic acid. Terchebulin showed good antibacterial activity, MIC =125 μg/ml and minimum bactericidal concentration (MBC) = 250 μg/ml. On the other hand ellagic acid showed good MIC value 125 μg/ml, with low MBC value 1000μg/ml. Gallic acid exhibited lipase inhibitory activity with IC_{50} value of 149.3 μM, which showed strong inhibition compared with terchebulin, IC_{50} 260.7 μM. However all compounds exhibited better or equal DPPH radical scavenging activity to (+)-catechin as positive control. Ellagic acid and terchebulin showed the best DPPH radical scavenging activities, IC_{50} 4.86 μM and 4.90 μM respectively. This study demonstrated that terchebulin possess a potentiality as anti-acne agent.

In chapter four, *A. nilotica* pods methanol extract was elected for further separation due to there outstanding antioxidant activity and good lipase inhibitory activity. In the last years evidence supports a pivotal role for cellular inflammatory events at acne lesion development. The emphasis has moved from acne as a primarily hyperproliferative disorder of the sebaceous follicle to that of an inflammatory skin disorder. From previous reports about *A. nilotica* pods showed a good anti-inflammatory activity using *in vivo* model. So we speculate that may *A. nilotica* playing role for reducing acne inflammation. Based on these combine activities, the extract was subjected to the same method of separation and isolation we mentioned before in chapter Three. Two compounds well known were isolated included methyl gallate and gallic acid. The methyl gallate was demonstrated excellent antioxidant activity (IC_{50}= 3.81 μM).

In conclusion, this study has reported for the first time a prospective anti-acne agent from Sudanese medicinal plants. Terchebulin from *T. laxiflora* wood possess a potentiality as anti-acne agent. Gallic acid from *T. laxiflora* and *A. nilotica* have potency as *p. acnes* lipase inhibitor.

In depth, studies at cellular and *in vivo* level would now be needed to approve these anti-acne activities of these compounds.

学　位　論　文　要　旨

アクネは毛包脂線に形成される角質タンパク質と脂質の混合物の蓄積に始まり、時に強い炎症を伴い、症状が進行した場合は瘢痕形成も認められる炎症性疾患の一つである。時には落胆やフラストレーションなど心身性の病気を引き起こすこともある。その直接的な原因菌として *Propionibacterium acnes* (*P. acnes*)の関与が広く知られている。*P. acnes* は、皮脂中のトリグリセライドを菌自身が産生するリパーゼによって分解し、生成した脂肪酸を資化することにより生育する。さらに酵素や活性好中球のような内在性のもから紫外線照射による外因性酸化促進剤蓄積の両方から生じる活性酸素種(ROS)によって誘導される脂質過酸化物がアクネ進行の原因の一つとして理解されている。

皮膚疾患に対する植物成分を用いた伝統療法は、現在でも患者達の中で親しまれて、広く用いられている。スーダンでは多くの人々によってプライマリーヘルスケアの源として伝統生薬が用いられているが、スーダン産薬用植物を用いた抗ニキビ剤の研究はほとんど行われていない。

本研究の第一の目的は、抗菌活性、リパーゼ阻害および抗酸化活性を持つスーダン産薬用植物エキスを見出すことである。第二の目的は、活性のある植物エキスから活性成分を特定することである。

まず 28 科に属する 40 種の植物からメタノールおよび 50%エタノールによる 104 種の粗抽出物を得、*in vitro* での抗ニキビ活性を測定した。それら抽出物のうちシクンシ科に属する種の抽出物が非常に良好な *P. acnes* に対する抗菌活性を示し、特に *Terminaria laxiflora* 材のメタノールおよび 50%エタノール抽出物は最も高い抗菌活性を有し、MIC（最小発育阻止濃度）が 130 μg/ml であった。*Abrus precatorius* 種の 50%エタノール水抽出物、*T. laxiflora* 材 50%エタノール水抽出物、*Acacia nilotica* 鞘のメタノール抽出物は 500μg/ml の濃度で 70%以上のリパーゼ阻害活性を示した。また、*Acacia nilotica* 鞘のメタノール抽出物は際だった DPPH ラジカル補足能力を示し（IC_{50} = 1.32μg/ml）、ポジティブコントロールの(+)-カテキンより高かった。選択した抽出物のフォーリンチオカルチャー法による総フェノール量、バニリン塩酸法によるフラバノール量、および BSA 吸着能によるタンニン量を定量したところ、*Terminalia brownii* 樹皮において最も高い総フェノール量を、*A. nilotica* において最も高いタンニン量を含むことを測定した。これ

ら成分分析の結果、*T. laxiflora* 材は加水分解性タンニンを、*A. nilotica* および *T, brownii* 樹皮には縮合型タンニンを多く含むことを示唆した。

抗ニキビ効果に関するスクリーニングの結果、*T. laxiflora* 材メタノール抽出物をその候補抽出物として選択した。活性成分を特定する目的で、本メタノール抽出物を ODS カラムによる中圧クロマトグラフィーおよび LH20 ゲルを用いたオープンカラムクロマトグラフィーに供した。メタノール、水およびアセトンを溶離剤に用いて分画し、活性成分を調べたところ、5 つのタンニン関連化合物、ellagic acid, flavogallonic acid dilactone, terchebulin および gallic acid を単離同定した。その中で、terchebulin は MIC が 125μg/ml, MBC が 250μg/ml で最も高い抗菌活性を示した。一方、ellagic acid も MIC が 125μg/ml であったが、1000μg/ml の MBC を示し、terchebulin に比べ殺菌性は劣ることが解った。リパーゼ阻害活性に関しては、terchebulin の IC_{50} が 260.7μM であったのに対し、gallic acid のそれは 149.3μM であった。また単離したすべての化合物は、ポジティブコントロールの(+)-catechin より高いかほぼ同程度の DHHP ラジカル補足能力を示し、特に terchebulin と ellagic acid は IC_{50} が 5μM 以下で相当強力な抗酸化剤と判断できる。以上の結果を総合的に考察すると、本研究で検討したスーダン産薬用植物の中では、terchebulin が抗ニキビ剤としての可能性を示唆した。

本研究で、*A. nilotica* 鞘メタノール抽出物は高い抗酸化性とリパーゼ阻害活性を有する事が明らかとなったが、これまでに他の研究者により、本抽出物は抗炎症作用を持つことが細胞試験を通して明らかとなっている。皮脂過剰増産性疾患に関する研究は、アクネ菌を除去する目的から皮膚の抗炎症作用に着目が移りつつある。そのような中で、試験管レベルではあるが、*A. nilotica* 鞘メタノール抽出物特に、methyl gallate と gallic acid に抗炎症作用が認められたことは、今後の我々の研究に大いに役立つ知見であると考える。

Chapter 1
General introduction, research objectives and thesis outline

1.1 Introduction

Acne vulgaris is one of the most common skin disorders with an estimated prevalence of up to 85% in the young population (Dreno and Poli, 2003; Wood, 1997). Acne is affecting nearly all adolescents and adults at some time in their life (Webster, 2002). The prevalence of the disease is higher among men than women, but women are more likely to seek medical advice for the acne treatment (Stern, 1992; Stern, 1996). Acne is a common problem worldwide and while some studies have suggested that there may be small racial differences in the relative prevalence of specific types of acne, and variations in the clinical manifestations of the disorder between different racial groups, in general acne vulgaris appears to be more or less equally prevalent among blacks, whites, and Asians. However, the natural history, clinical course, and most importantly, the long-term sequelae of acne can be considerably different. All darker-skinned individuals are at increased risk of postinflammatory hyperpigmentation. Acne vulgaris is an extremely common dermatological problem among Africans and people of African descent worldwide. Few studies of any of the major acne therapies have been carried out in exclusively black populations, and relatively little is known about the specific responsiveness of black skin to these agents (Jack, 2001).

There are economic value for acne pathogenesis by recognizing acne as a chronic disease similar to eczema, early and aggressive treatment can be started to avoid the psychological sequela that can result from active disease and scars (Thiboutot et al., 2009). For instance; acne causes significant morbidity and the direct costs associated with it exceed $2.2 billion per year of acne in the United States (U.S.) is estimated to exceed, with $100 million spent on over-the-counter products. Despite this high cost, 81 % of women report failures with systemic antibiotics and failures with isotretinoin

range from 15 to 30 % (George et al., 2008; Bhambri et al., 2009). The pathogenesis of acne is complex and includes abnormal keratinization, bacterial proliferation on the skin by *Propionibacterium acnes* and hyperactivity of the sebaceous glands. Fig (1.1) shows a schematic picture of a cross section of a pilosebaceous follicle. These follicles are found in highest amount on the face and upper trunk.

The beginning of acne typically appears during adolescence, with the development of non-inflamed spots (whiteheads and blackheads), which often, but not always, are found on the forehead and mid-facial region. They are called comedones. These superficial spots can become inflamed and are then called papules and pustules. Very large, deep inflamed spots are called nodules. In the clinic acne is divided into mild, moderate or severe (Fig 1.2) and the choice of treatment between these are differs. Acne usually begins at puberty, when the output of sebum by tiny hair follicles in the face and upper trunk increases substantially (Rothman and Lucky, 1993).

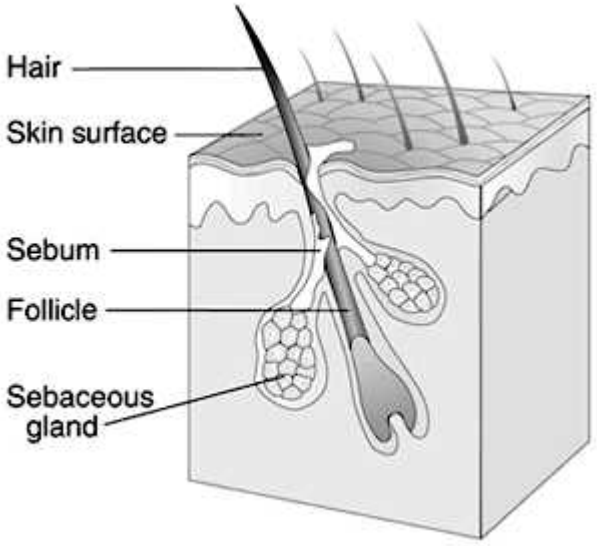

Fig 1. 1. Schematic picture of the pilosebaceous follicle

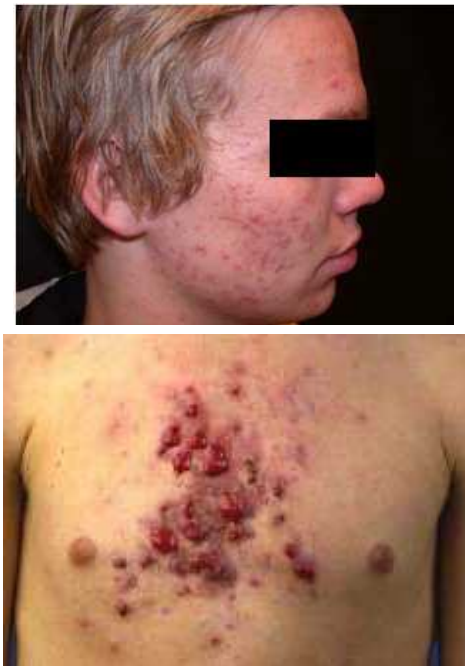

Fig 1. 2. Moderate acne in the face (up) and severe acne on chest (down)

(Photos by Camilla, 2009)

The production of sebum is controlled by male hormones (androgens) in both sexes. Genetic factors play an important role. There are families which have several individuals with severe forms of acne (Zouboulis et al., 2005).

There are four important pathophysiological factors contributing to the acne development: sebum secretion, comedo formation, ductal colonization with *P. acnes,* inflammation and immunological host reactions (Fig 1.3). The pilosebaceous follicle is the target organ in acne, and the earliest morphological change in the pilosebaceous unit is abnormal follicular epithelial differentiation (Knutson, 1974). This process is called comedogenesis, the growing comedo eventually blocks the flow of sebum (Cunliffe et al., 2004). The bacterium produces an extracellular lipase that hydrolyses sebum triglycerides to glycerol, used by the bacterium as a growth substrate and free fatty acids, which have proinflammatory and comedogenic properties (Shalita and Lee, 1983).

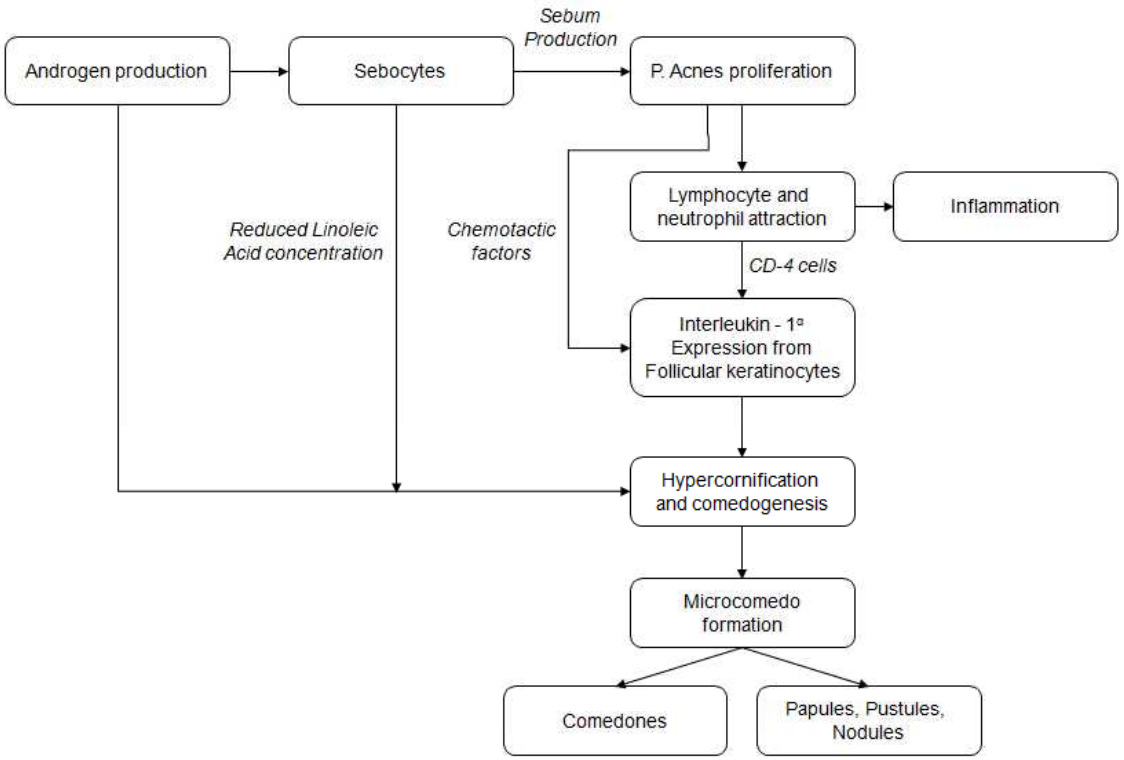

Fig 1. 3. The pathogenesis of acne vulgaris (leyden, 2003)

1.1.2 *Propionibacterium acnes* in acne formation

P. acnes is a non-spore-forming, Gram-positive anaerobic. Although it is usually regarded as a strict anaerobe, it can tolerate oxygen up to 100% saturation but grows at reduced rates (Cove et al., 1983). *In vitro*, *P. acnes* is capable of surviving for as long as 8 months under anaerobic conditions without subculture, suggesting that it could also survive in human tissues at low oxidation potentials (Csukas et al., 2004). In addition, *P. acnes* is slow growing and can resist phagocytosis and persist intracellularly within macrophages (Webster et al., 1985). This resistance to phagocytosis may be attributable to the organism's complex cell wall structure, which also has a surface fibrillar layer (Montes and Wilborn, 1970)

P. acne is not pathogenic by normal standards because it is present in nearly 100% of healthy persons (Jappe et al., 2002). It is isolated from the skin surface, but the

multiplication takes place in the duct of pilosebaceous follicles. The bacterium is generally 0.5-4 nm in size, dome-shaped and beige to pink in color (Fig1.4). Only 17% of the follicles in normal individuals are colonized (Eady and Ingham, 1994). The density varies a lot among individuals and between different sites in the same person. Levels are highest in areas that are rich in sebaceous glands, such as the face and scalp. The bacterial population correlates with the amount of lipids produced in different body regions (McGinley et al., 1980). These findings suggest that sebum serves as a substrate for *P. acnes* growth. A study on *P. acnes* strain populations in the human skin microbiome associated with acne demonstrated that although the relative abundances of *P. acnes* were similar, the strain population structures were significantly different in the two acne patients and healthy individuals. Certain strains were highly associated with acne, and other strains were enriched in healthy skin (Fitz-Gibbon et al., 2013).

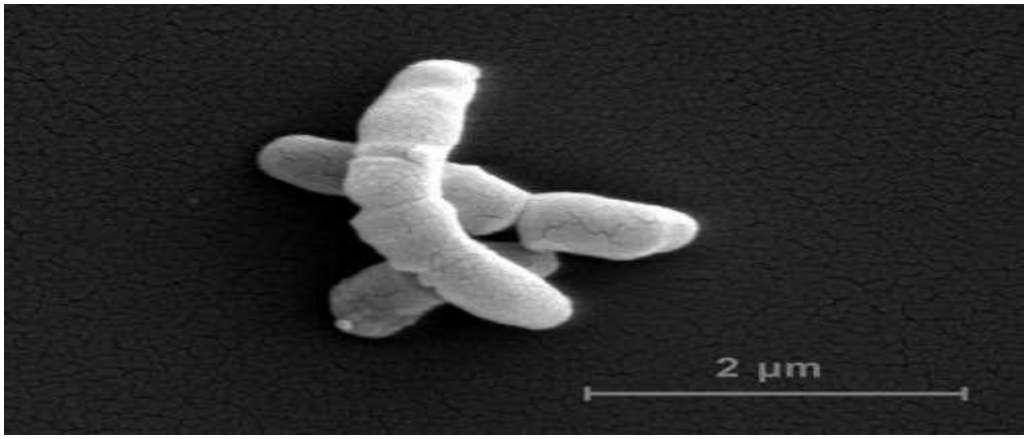

Fig 1.4. Electron microscopy image of *P. acnes*

(Photos by Camilla, 2009)

P. acnes bacteria display a variety of immunomodulatory properties. Also is a strong stimulant of pro-inflammatory mediators released by phagocytic cells, especially of tumor necrosis factor TNF-α (Simpson, 2001). Although *P. acnes* and

Staphyloccocus epidermidis are two major bacterial strains isolated from acne lesions. Nevertheless, only *P. acnes* seem to be implicated in the pathogenesis of inflammatory acne vulgaris. *P. acnes*, but not *S. epidermidis*, evoked mild local inflammation of infected mice ears. Macrophages elicited with *P. acnes* produced more tumor necrosis factor TNF –α and interleukin IL-12 than those induced with *S. epidermidis*, while *S. epidermidis* was a stronger inducer of IL-10 production. Both bacteria equally induced the generation of nitric oxide (NO) and reactive oxygen species (ROS). In contrast, only *P. acnes* showed adjuvant properties. *S. epidermidis*, in contrast to *P. acnes*, does not exert pro-inflammatory properties. Thus it is unlikely that *S. epidermidis* may be implicated in the pathogenesis of inflammatory acne vulgaris (Biatecka et al., 2005). *P. acnes*, by acting on Toll-like receptors (TLR-2), may stimulate the secretion of cytokines, such as interleukin (IL)-6 and IL-8 by follicular keratinocytes and IL-8 and -12 in macrophages, giving rise to inflammation. *P. acnes* may induce an immunological reaction by stimulating the production of sebocyte and keratinocyte antimicrobial peptides, which play an important role in the innate immunity of the follicle (Kurokawa et al., 2009).

1.1.2.1 Antibiotic resistance in *P. Acnes*

The use of antibiotics in acne vulgaris inhibits the growth of *P. acnes* and⁄or their production of pro-inflammatory mediators (Tan, 2003). Until the late 1970s the propionibacteria were uniformly susceptible to antibiotics (Leyden et al., 1975). By 1979 the situation had changed. Resistance to macrolide, erythromycin, lincosamide and clindamycin has been reported among cutaneous propionibacteria from Europe, USA, Australia and the Far East (Leyden et al., 1983; Ross et al., 2001). There are few reports of propionibacterial resistance to tetracycline.

Although the exact cause of *P. acnes* resistance to antibiotics is not fully understood, it can be related to the inability to obtain *in vivo*, in the sebaceous follicle of acne patients, a concentration of antibiotic greater than the minimum inhibitory concentration. *P. acnes* resistant to antibiotics may be also related to poor treatment compliance, and easy access to therapeutic agents without medical supervision. Prolonged use of antibiotics is also one of the factors that have contributed to the development of antibiotic resistance (Abdel Fattah and Darwish, 2012).

Current anti-acne treatment is based on the use of retinoids or benzoyl peroxide, frequently in combination with antibiotics like clindamycin, tetracycline and erythromycin. However, repeated use of these agents is known to induce antibiotic resistance in acne causing organisms (Nishijima et al., 2000). Due to increasing of *P. acnes* resistance, side effects and sometimes high cost of treatment, interest in medicinal herbs has been progressively increased on the last decade.

It is found that while treating acne vulgaris, only antimicrobial approach does not work. The organisms involved more than one pathological condition; hence the treatment of acne needs broader approach.

1.1.3 Lipase in acne formation

The multiplication of *P. acnes*, the overproduction of sebum, and follicular hyperkeratinization are three consequential physiological factors in the pathogenesis of acne. Numerous enzymes produced by *P. acnes* such as lipase, protease, hyaluronidase and acid phosphatase contribute to the spread of acne (Jappe, 2003). Lipase hydrolyses sebum triglycerides releasing irritating free fatty acids in the pilosebaceous follicles, which is one of the major causes of lesions in acne vulgaris (Puhvel et al., 1975; Strauss

and Kligman, 1960). Earlier studies have shown that intradermal injection of free fatty acids induces inflammation by stimulating the follicular epithelium (Lee, 1982; Strauss and Pochi, 1965). A 40% reduction in free fatty acids was observed among acne patients treated with lipase inhibitors such as halopyridyl phosphorus compounds (Weeks et al., 1977). In addition, di-isopropyl phosphofluoridate completely inhibited lipase as well as polymorphonuclear leukocyte chemotaxis (Lee et al., 1982). Furthermore, antibiotics such as tetracycline, erythromycin and propylene phenoxetol have been shown to inhibit *P. acnes* lipase activity (Uncles and Gemmell, 1982). A decline in free fatty acids caused by lipase inhibition is associated with a decrease in the growth of *P. acnes* (Strauss and Kligman, 1960). Because antibiotic resistant species of *P. acnes* showed higher lipase production, this enzyme could be considered as an effective pharmacological target for acne vulgaris (Higaki, 2003). *P. acnes* lipase itself can act as a chemotactic factor (Lee et al., 1982), and it is likely that other contributing factors are involved. In addition, we should not ignore the existence of acid phosphatase, hyaluronate and protease which are extracellular enzymes produced by *P. acnes* (Holland et al., 1981). These enzymes may also be of importance in inflammatory acne among the enzymatic activities. *P. acnes* lipase (GehA, glycerol-ester hydrolase A) has been recognized as one of the virulence factors involved in the pathogenesis of acne (Higaki, 2003). GehA enzyme is the main responsible for the hydrolysis of sebum triacylglycerides, thus releasing glycerol and free fatty acids. Glycerol is a source of nutrients for *P. acnes*, whereas fatty acids are highly inflammatory, chemotactic, and irritating for the sebaceous follicle cells (Jappe, 2003; Toyoda and Morohashi, 2001). Moreover, fatty acids favour ductal hypercornification by adhesion and packaging between keratinocytes and increase adhesion between *P. acnes* cells and between *P.*

acnes cells and follicle cells, which favours *P. acnes* colonization and biofilm formation (Gribbon et al., 1993). Furthermore, GehA itself is a strong chemotactic and pro-inflammatory antigen (Lee et al., 1982). The effect of several natural substances on GehA has been evaluated revealing that glycyrrhizic acid, (±)-catechin and kaempferol are promising candidates for the treatment of acne due to their strong inhibitory activity on GehA, as well as to their other anti-acne effects and their low toxicity. Also extract from *T. chebula* showed significant inhibition of lipase activity and number of *P. acnes*.

1.1.4 Reactive oxygen species in acne formation

Oxygen, which is an important and vital component for human, can produce reactive types (superoxide anion, hydrogen peroxide, and hydroxyl radicals) known as ROS. These radicals are formed with the reduction of oxygen to the water. Normally, the production of these radicals is slow and they are removed by the antioxidant enzymes existing in the cell. In acne, sebum produced by sebaceous glands, content of sebum changes and reactive oxygen species (ROS) may be released from the impacted damaged follicular walls; at the same time it is thought that this may be the reason for the progress of the inflammation in the pathogenesis of the disease. It is also known that some of the drugs used commonly in the treatment of acne function by decreasing ROS (Arican et al., 2005). Superoxide dismutase (SOD), catalase (CAT), and glucose-6-phosphate dehydrogenase (G6PD) are some of the important antioxidant enzymes and have been reported to significantly lower in the blood of acne patients (Katzman and Logan, 2007). Malondialdehyde (MDA) is the end product of lipid peroxidation and one of the indicators of oxidative stress. When SOD and CAT enzymes are insufficient for oxidative stress, ROS denotes its impact by starting the

lipid peroxidation on the membranes of organs and cells MDA increase (Basak et al., 2002). However, the low activity of SOD in polymorphonuclear leukocytes may be responsible for the increased levels of superoxide anion radicals in the epidermis.

There are herbs may promisingly to inhibit the oxidative mediators but only this effect may not be sufficient for the treatment of acne. These herbs may be useful only in combination with anti-inflammatory and antimicrobial herbs; or the herb having multiple actions can be more effective because it is proved that combination therapy for acne vulgaris is much more effective than one with single drug (Leyden, 2003).

(ROS) are subsequently generated from the hypercolonization of *P. acnes* (Leyden, 2001) in addition to metabolism in living organisms and from UV exposure. Although ROS perform a useful function in the skin barrier against acne microbes (Cals-Grierson and Ormerod, 2004), excess formation affects skin condition by activating neutrophil infiltration. ROS including lipid peroxide and NO play an important role in inflammatory acne as well as in tissue injury. ROS stimulate the formation of nuclear factor *k*B (NF-*k*B) (Gougerot-Pocidalo and Revillard, 1993), promote TNF formation (Trefzer et al., 1993) and consequently activate T lymphocytes and keratinocytes. The cytokines IL, TNF, Interferons (IFN), transforming growth factor (TGF) and prostaglandin (PG) are then produced and released causing microcomedones (Wilmer et al., 1994; Pentland and Mahoney, 1990). The resulting microcomedones further develop into comedones and inflammatory lesions.

Grange et al., 2009 stated that keratinocytes are not mere targets of the innate immune response but are directly involved in the defence mechanisms aiming at eliminating pathogens. In response to *P. acnes*, keratinocytes can produce massive

amounts of ROS that in return inhibit bacterial growth (Fig 1.5). Those ROS do not only eliminate the bacteria but also generate inflammation. Thus, They assumed that the severity of acne depends on the balance between the ability of the *P. acnes* strain to induce a potent immune response (Nagy, 2005) and the capability of the host to generate and to detoxify the ROS produced (Akamatsu et al., 2003; Abdel Fattah et al., 2008). Therefore, inhibiting this inflammatory reaction using appropriate antioxidant molecules could be considered as a potential treatment of acne. In acne vulgaris the antioxidant defence system is damaged, and thereby antioxidant drugs can be indicated for treatment of acne. Topical application or oral supplementation of antioxidants appears to be an effective approach in enhancing the activity of therapeutic agents or avoiding their adverse effects (Sarici et al., 2010).

1.1.5 Sebaceous Gland in acne formation

The human sebaceous gland is a multi-acinar, holocrine- secreting tissue present in all areas of the skin except for the palms and soles (Benfenati and Brillanti, 1939). The number of sebaceous glands remains approximately constant throughout life, whereas their size tends to increase with age. (Zouboulis et al., 2001).The most obvious function of the sebaceous gland is to excrete sebum. Additional functions of the gland are associated with the development of acne like: synthesis of specific lipids with antimicrobial activity (Wille and Kydonieus, 2003) and exhibition of pro- and anti-inflammatory properties (Böhm M et al., 2002).

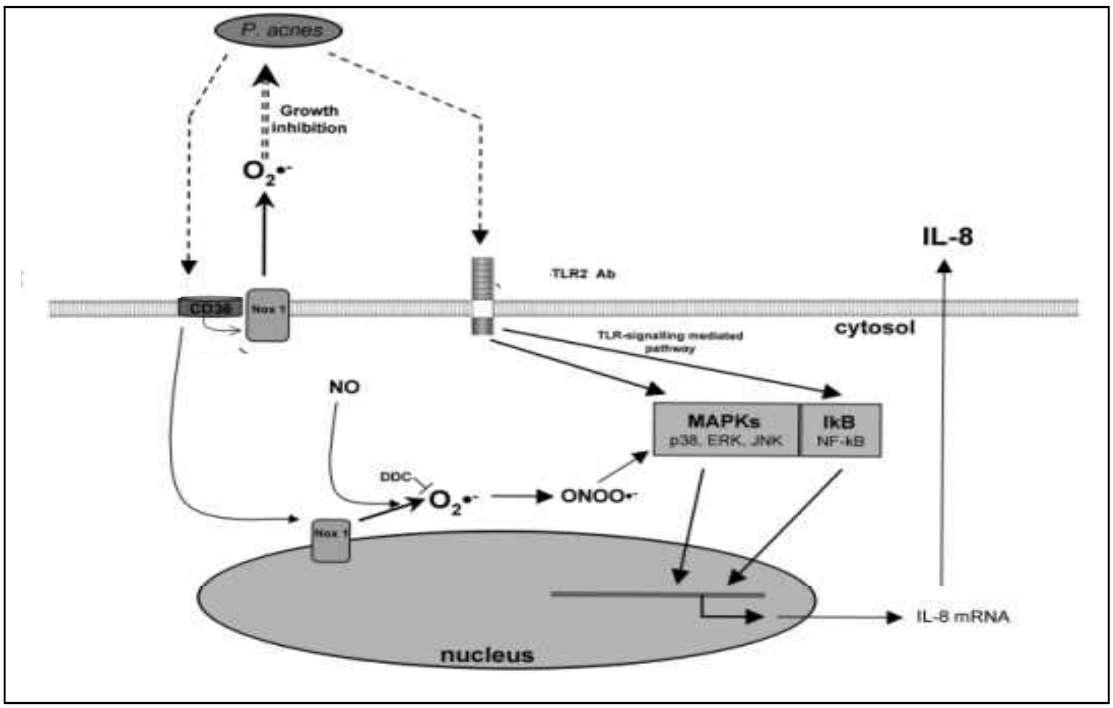

Fig 1.5. Proposed molecular mechanisms through which *P. acnes* induces ROS and IL-8 production in keratinocytes. Surface components of *P. acnes* are recognized by both CD36 and TLR-2. CD36 triggers the production of $O_2^{\bullet-}$ through the NADPH oxidase pathway (NOX) and combines with NO to form peroxinitrites which, in turn, activate p38 and ERK MAPKs, thus contributing to IL-8 production. In parallel, IL-8 production is activated through the TLR-2 signalling pathway (Grange et al., 2009).

The sebaceous gland is an important formation site of active androgens. Androgens are well known for their effects on sebum excretion, Estrogens, glucocorticoids and prolactin also influence sebaceous gland function (Christos and Zouboulis, 2004). Androgens, testosterone and its highly active metabolite dihydrotestosterone play a role in the development and progression of acne. Furthermore, 5 alpha-reductase (type 1 ,type 2) which catalyze the conversion of testosterone to dihydrotestosterone, has been a target of manipulation in the treatment of acne hence synthetic 5 alpha-reductase activity inhibitors have shown therapeutic promise (Chen et al., 1996). In human studies, green tea decreased sebum production. Tea gallates and α-linoleic acid have selective inhibitory activity against 5α- reductase. (Mahmood et al., 2010)

The secreted sebum normally contains a mixture of lipids, squalene, wax and cholesterol both in free and in ester forms and triglycerides that naturally provide a skin barrier function (Pilgram et al., 2001). However, the resulting abnormalities in sebaceous glands because of hormonal effects alter sebum composition and linoleic acid content is notably decreased (Downing et al., 1986). Thus, the skin barrier is impaired and colonization of normal flora is promoted.

Linolenic acid also has anti-inflammatory affects on our skin and helps boost our immune system (Letawe et al., 1998). There was significantly effect of topically applied linolenic acid on the size comdones (Fig 1.3), an almost 25% reduction in their over size being achieved over 1 month treatment period. In contrast, no change was found in placebo- treated sites. It is concluded that topical linoleic acid might play a role as acomdolytic agent in acne -prone patients (Diezel et al., 1993).

1.1.6 Herbs for acne treatments

The use of natural remedies is a highly approached in human health (Bent and Ko, 2004), in particular cosmetics with an ongoing search for novel biologically active botanical agents (Schürch et al., 2008). There are plants that currently used and those with a high potential to treat acne, include: *Aloe vera* extract is used as a component in Ayurvedic formulations. It significantly reduced acne lesions (Lalla et al., 2001). This Asian dermatological remedy was in accord with the therapeutic use of Aloe spp. in South Africa (Grace et al., 2008). However, *A. vera* was insignificant to suppress *P. acnes*-induced ROS and proinflammatory cytokines (Jain and Basal, 2003). In the same ayurvedic formulation, *Azadirachta indica*, *Curcuma longa* and *Hemidesmus indicus* were used for acne treatment (Lalla et al., 2001). These herbs significantly suppressed the production of ROS induced by *P. acnes* (Jain and Basal, 2003). Accordingly, their anti-inflammatory activity could be stronger than *A. vera*, highlighting their potential in inflammatory lesions treatment. A common spice, poultice onion (*Allium cepa*), was traditionally used for acne (Griffith et al., 2002) owing to its mild keratolytic, anti-fungal and bacteriostatic properties with respect to its sulphur containing (Gupta, and Nicol, 2004) including its anti-inflammatory flavonoids (Dorsch, 1996). However, its malodor limits the application as well as the possibility of irritation.

Asia is not the only continent using traditional herbs for the treatment of acne vulgaris. *Centella asiatica* was used as a general tonic for leprosy and wounds particularly for acne in Africa (Van Wyk, 2008). Although its mechanism remains unknown, skin care products containing *C. asiatica* are widely commercialized in Asia. *Rosa damascene*, which is mostly used as a fragrance, was found to effectively inhibit *P. acnes* with respect to its anti-inflammatory action (Tsai et al., 2010). Similarly, rose oil

was used in the treatment of acne (Stevensen, 1998). Therefore, rose should be incorporated into cosmetic products as a multifunctional ingredient. However, an appropriate delivery system should be developed to impart their efficacies in addition to the standardization of these herbs. Furthermore, an optimized and effective dose should be evaluated prior to the development of preparations in order to avoid irritation or allergy in subjects with hypersensitive skin. Strict quality control will ensure their safety and efficacy. In addition, combination treatment should be conducted as it was found to be more effective than the application of a single product with regard to synergistic effects on the pathogenesis of acne (Kanlayavattanakul and Lourith, 2011).

1.2.1 Traditional Sudanese medicine

Sudan is the third largest country in Africa, with diverse climate conditions and different ethnic communities, holds the potential of an immense wealth of flora with a variety of uses in traditional medicine. Many of these plants have been used by herbalists for different purposes.

Sudan encompasses different terrains and climatic zones, ranging from desert and semi-desert in the north to equatorial with a short rainy season (semi-arid and semi-humid) in the centre to equatorial with a long rainy season (arid-humid and equatorial-humid) in the south. Thus, the range of mean annual rainfall (m.a.r.) is expected to vary in accordance with the climatic zones. It ranges from zero to heavy (1400 mm). Depending on the climatic zones and the amount of rainfall, the type of soil and cultivation practices are usually classified as one of four categories ranging from humid and rainy climate (Ferrasols, Nitosols and Vertisols types) to arid climate (Yermosols poor soil types). This climatic variation has a direct impact on the immense

diversity of vegetation. Based on the variation of climate, White (1983) divided Sudan into five main vegetation regions: Desert, Semi-desert scrub and grassland, Thorn savanna and scrub, Deciduous savanna woodland and Flood region.

Sudanese folk medicine represents a unique blend of indigenous cultures of Islamic, Arabic and African traditions. Consequently, treatments exist for a variety of diseases, both epidemic and endemic. To face these diseases, people have tapped the environmental resources, e.g. plants, minerals and animal products for the management of their health (El-Hamidi, 1970). For example Dukhan is popular custom especially favoured by Sudanese women for sexual enhancement, cleanliness and also medical purposes. In this custom, Sudanese women use the smoke from different woody plant species like: Habil wood (*Combretum ghasalense*), Subakh (*Terminalia brownii*) and Talih (*Acacia seyal*) to fumigate their naked bodies while covering themselves with a sheet of cloth in the form of a tent. The main purpose is to impart fragrance into the skin by long exposure to the smoke of fragrant woods (Baser and Abeywickrama, 1995: Vasisht and Kumar, 2006). In this respect, the Sudanese have amassed a large body of curative methods, techniques and recipes. Though not yet investigated systematically or in depth, there are clues in literature about the bioactivity of medicinal plants and their chemical constituents. Sudanese medicinal plants have been reported to exert antimicrobial activity against viruses, bacteria, and protozoa (El-Tahir, 1999; Ahmed El-H et al., 2010). As infections with worms or molluscs represent a common affliction in that area, medicinal plants have been considered for treatment of these infections (Koko et al., 2000; Koko et al., 2005). Immunomodulatory properties of Sudanese medicinal plants have also been observed (Koko et al., 2008).

1.2.2 Overview of medicinal plants in Sudan

The flora of Sudan consists of 3137 documented species of flowering plants belonging to 170 families and 1280 genera. It is estimated that 15% of these plants are endemic to Sudan. The intersection of cultures and the unique geographical position of Sudan hold great potential for research in many fields, the most important of which is medicinal and aromatic plants. The diversity of climates in Sudan results in a rich variety of flora species corresponding to the wide range of ecological habitats and vegetation zones. In Sudan, it is a common practice to collect medicinal plants from their natural habitats for home consumption and export. Plants collected from different localities or geographic regions may have different chemical compositions. This may be explained by differences in climate, temperature, rainfall, altitude, day length and UV-radiation, all of which play an important role in plant development and affect the biosynthesis of secondary metabolites with biological activity (Khalid et al., 2012).

1.2.3 Use of medicinal and aromatic plants in traditional medicine in Sudan

Like many other countries, Sudan has a long tradition of folk medicine. Indigenous remedies are often the only form of therapy available to poor people living in rural areas or cities. It has been estimated that only 11% of the population has access to formal health care. The governmental medical services, which were free of charge in the past, are now available at high charges. Poor people frequently cannot afford medicines. Since medical care is not available or is too expensive, the majority of people depend on traditional remedies prescribed by unregistered traditional healers (Arcury et al., 2009)

Medicinal and aromatic plants and their derivatives represent an integral part of the life and culture of the people of Sudan. This is not only true for treatment of human

diseases, but also for veterinary use (Omer and Adam, 1999: Bakhiet and Adam, 1995). Because of the tremendous importance of medicinal plants for human and animal health, research on valuable pharmacological effects and possible unwanted side effects or toxicity is required to improve the efficacy and safety of Sudanese herbal medicine. In Table (1.1) of this review, I provide an updated overview of traditional uses the selected Sudanese medicinal plants, which are mentioned in this study (Khalid et al., 2012; Ebrahim et al., 2012).

Table 1.1. A brief review of the selected Sudanese medicinal plants and their use in traditional medicine

No. Scientific name	Local name	Used part	Traditional uses
1- *Parkinsonia aculeata*	Sesaban	Leave	To treat fever and malaria
2- *Abrus precatorius*	Habat Alarous	seed	Against chronic nephritis and diabetes
3- *Trigonella foenum-graecum*	Hilba	seed	The seeds are used as antidiarrheal, anti-spasmodic, anti-amoeba dysentery and anti-diabetics. The seeds are also use as a food additive and to increase secretion of lactating mothers and to facilitate expulsion of the placenta
4- *Ambrosia maritime* L.	Damsisa	Arial parts	The herbs are used in treatment of urinary tract infections and elimination of kidney stones, whereas the leaves are used as anti-diabetic and anti-hypertensive
5- *Vernonia amygdalina* Del.	Gharib elwadi	Leaves	Antimicrobial ,tonic and anti-malaria

6- *Aristolochia bracteolata* LAM	Um Galagil	Arial parts	Treat malaria and HIV-1
7- *Citrullus coloynthis* (L.) Schrad	Al-handal	Dry Fruit	A gram of fruits used as a gastro-intestinal stimulant or irritant, anti-rheumatic, for treatment jaundice, various types of cancer and snake bites
8- *Ziziphus spina –christi* (L.) Desf.	Alsidir	Fruit Leaves Bark	3 g of the leaves are crushed and used as a poultice for hair tonic (shampoos and other cosmetic preparations)
9- *Lawsonia inermis* Linn	Henna	Leaves	Maceration of leaves is used as an anti-bacterial and anti-fungal , for skin diseases ,alopecia and anti-pyretic
10- *Moringa oleifera*	Shagarat al rawag	Leaves	Antimicrobial, antitumor, febrile and pulmonary diseases
11- *Salvadora persica* L.	Arak	Stem Leaves	The branches and roots are used for brushing teeth Gingivitis, malaria, liver swellings and HIV

12- *Tamarix nilotica*	Tarfa al Nile	Stems	Febrile, colds also the stem use for hemorroids
13- *Calotropis procera*	Ushar	leaves	Against scorpion bites and jaundice, healing thorn injuries and anti-rheumatic
14- *Solenostemma argel* Hayne	Harghel	Leaves	Maceration of 2 g of the dried aerial parts is used for cough, gastro-intestinal cramps and urinary tract problems, carminative and antispasmodic
15- *Xanthium brasilicum* W.	Ramtouk	leaves	Antioxidant and anti-malaria
16- *Lepidium sativum*	Hab el Rashad	seed	1.5 g of the poultice of seeds for the treatment of Madura foot .Treat diarrhea and anti-asthmatic
17- *Ammi visnaga L.*	Khella Baladiya	Fruits	Decoction of 3 g of fruits is used for renal urethra stones and smooth muscle relaxant

18- *Khaya senegalensis*	Mahogany	Bark	Anti-malarial, against hepatic inflammation , sinusitis, skin diseases, treat malaria and relieve toothache
19- *Balanites aegyptiaca*	Laloub	Bark leaves Fruit wood	Mesocarp of fruits is eaten or macerated and used as a laxative and anthelmintic. Oil of the seeds is topically used for wound healing. purgative, treat jaundice and malaria
20- *Carum carvi*	Karawiya	Fruits	Diuretic, antispasmodic, carminative and mainly for stomach complaints in both adult and children.
21- *Hibiscus sabdariffa* L.	Karkadeh	flower	Maceration or infusion of 5 g of the fruits epicalyces is used an anti-hypertensive, diuretic, anti-microbial, anti-spasmodic and for relaxation. Treat hypertensive, colds and fever
22- *Abutilon pannosum*	Hamboak	Leaves	hepatoprotective, antiplasmodial and hypoglycemic activities
23- *Terminalia brownii*	Sobagh- Subaraya	Bark Wood	Fumigants are used to treat rheumatic and back pains.

24- *Combretum hartmannianum* (Schweinf)	Habeil Al Gabal	Bark Wood	arthritis rheumatism , treat dryness of skin, bacterial infection
25- *Terminalia laxiflora* Engl& Diels	Subagh – Darut	Wood	fumigation ingredient
26- *Guiera senegalensis* J.F.Gmel	Gubeish	Leaves	Infusion of 10 g of the leaves is used for the treatment of bronchitis, fever, cough and stomach complaints, improve the health of hyper-tension and diabetic patients. To treat febrifuge and hypertension
27- *Acacia seyal* var seyal	Talih Ahmer	Bark Wood	Stem fumigant is used against rheumatic pain
28- *Acacia tortilis F*orssk (Hayne)	Seyal, samo	Bark Wood	diarrhoea and stomach-ache
29- *Acacia seyal* var fistula	Sufar abiad	Bark Wood	Bacterial infection of the skin ,dysentery and stem fumigant is used against rheumatic pain

30- *Acacia nilotica* (L.) *Gard*	El-garad	Pods bark	Anti-inflammatory, maceration of 2 g of pods is used for pneumonia, malaria and as a gargle for tonsillitis. Powder of fruits is used to treat diarrhoea and dysentery. Fumigation for colds and fever
31- *Haplophyllum tuberculatum* Forsk	Haza	Aerial part	Infusion of 5 g of the leaves is used as an anti-spasmodic, anti-diarrhoea and also used for the treatment of the prostate
32- *Kigelia africana (lam.)B*	Um shutoor	Fruit	Swollen mastitis and anticancer treatment
33- *Hyphaene thebaica*	Dom	fruit	10 g of a powder of fruit epicarps is used for the treatment of gastrointestinal ailments, wounds anti-inflammatory and anti-hypertensive. Antibacterial in eyes infection
34- *Moringa oblongifolia*	Irig al Mahaba	Stem	Anti-malaria
35- *Capparis decidua*	Taundub	Stem	Against jaundice, to treat swelling, against headache and anti-rheumatic

36- *polygonum glabrum*	Altomsahia	Leaves	anthelmintic and anti-inflammatory
37- *Fagonia cretica L.*	Umm Shuwaika	Aerial part	Against muscular pains, antispasmodic, anti-purgative, against heart burn and skin allergy
38- *Nigella sativa*	Alkamoon Alasod	seed	2 g of the seeds are used to treat many diseases, such as diabetes, hypertension, abdominal ulcers, prostate gland inflammations and anthelmintics
39- *Solanum dubium*	Gubbain	fruit	Treat wounds and skin tumors as dressing
40- *Grewia tenax* (Forsk)	Guddeim	seed	Maceration of 10 g of the fruits is used for the treatment of general fatigue and iron deficiency ammonia anaemia

1.3 Research objectives and thesis outline

The present study was undertaken with the primary objective of finding Sudanese medicinal plants which have anti-acne potency based on antibacterial, lipase inhibition and antioxidant activity. A second objective was to determine the active compound for anti-acne activity from these medicinal plants.

The study is divided into three chapters. Chapter two describes the screen process of selected Sudanese medicinal plants for anti-acne activity. In the subsequent chapter (Chapter three) characterize the anti-acne activity of tannin-related compounds isolated from *Terminalia laxiflora*. Finally, Chapter four presents the potency of *Acacia nilotica* pod extracts as anti-acne agent and there isolated compounds and general discussion.

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Chapter 2
Screening of anti-acne activity of selected Sudanese medicinal plants

2.1 Introduction

Acne vulgaris is a common skin disease of humans caused by bacteria inducing non-inflammatory and inflammatory skin lesions (Leyden, 2003). *P. acnes* has been recognized as an obligate anaerobic organism which is usually found as a normal skin commensal. This organism has been implicated over other cutaneous microflora in contributing to the inflammatory response of acne. It acts as an immunostimulator which can produce a variety of enzymes and biologically active molecules involved in the development of inflammatory acnes. These products include lipases, proteases, hyaluronidases, and chemotactic factors (Toyoda and Morohashi, 2001). Current treatment for acne is mostly based on antibiotics such as clindamycin and tetracycline derivatives. However antibiotic-resistant for *P. acnes* are widely spread and become a critical problem worldwide (Stathakis et al., 1997). During this decade there have been extensively studied for new compounds from natural substances possessing antibacterial activity against acne–inducing bacteria (Kumar et al., 2007)

Reactive oxygen species (ROS) are subsequently generated from the hyper-colonization of *P. acnes* and from ultraviolet exposure (Leyden, 2001).Therefore, substances that inhibit the growth of skin microorganisms and have anti-oxidant activity are of great interest, since they may cure or prevent various diseases related to the effects of free radicals and may be useful in the treatment of adult pimples.

The unique and diverse flora of Sudan and its prominent traditional medicinal plants could probably be a rich source of bioactive compounds (El-Kamali and Khalid, 1996). Information about Sudanese folk medicine was documented during comprehensive ethnobotanical investigations (El-Kamali and El Khalifa, 1999). In this

chapter, the first anti-acne screening of selected Sudanese medicinal plants is shown by antibacterial activity against *Propionibacterium acnes*, lipase inhibition and antioxidant activity.

2.1.1 Traditional knowledge of some Sudanese medicinal plants used in this study

2.1.1.1 *Terminalia brownii* Fresen

Terminalia brownii Fresen is known in Sudan as (Subagh – Subaraya). It's a leafy deciduous tree with an attractive some what layered appearance. It is native to Ethiopia, Eritrea, Kenya, Tanzania, Somalia, Sudan and Uganda. Usually 4-15 m high with a rounded, flat topped, spreading crown and straight bole; branches reaching close to the ground. Young bark are smooth and whitish changing to grey longitudinally fissured when get older. Young shoots are densely haired. Leaves are spirally arranged, crowded at the ends of branches (hence the generic name Terminalia), underside with bright hairs. Flowers are long white to cream, glabrous, calyx lobes acuminate and unpleasantly scented. Fruits are winged, smooth, and greenish when young, purplish – red to brown when mature. Seeds are delicate having two wings; the color of seeds is red to purple (Fyhrquist, 2007).

T. brownii is found in many parts of Africa and it has different uses. Ethnomedical information revealed that, traditional healers in Tanzania use leaves to treat diarrhoea and stomachaches, gastric ulcers, colic, heartburn (Mbuya et al., 1994). This medicinal plant is very much used by traditional healers in Kenya to treat various disease conditions such as malaria and paludism (Heine B and Heine I, 1988). The decoction of stem bark, trunk and branches is taken orally to treat dysmenorrhoea, nervosity, hysteria, epilepsy, beriberi, dyspepsia, stomachache, gastric ulcers, colitis

(Lindsay and Hepper, 1978; Mbuya et al., 1994). Leaves are used as diuretics and for the treatment of cut wounds. Stem barks are chewed to treat cough and as emetic, infusion of barks and leaves are mixed with meat to treat hepatitis (Timberlake, 1987)

In the Democratic Republic of Congo they use barks from the stems, branches, and trunks to treat urogenital infections, urethral pain, endometriosis, cystitis, leucorrhoea, syphilis, and gonorrhoea (Dhetchuvi and Lejoly, 1990). Traditional healers in Ethiopia use stem and bark to treat jaundice, hepatitis, liver cirrhosis (Heine and Brenzinga, 1988; Kokwaro, 1976; Wilson et al., 1979).

In Sudan *T. brownii* was classified as threatened medicinal plants. It occurs in Lowland Plains in Low Rainfall Savannah. The tree flower from April to June and fruiting occurs from October to November in Sudan (Orwa et al., 2009). It is a valuable timber tree and is widely used as a fumigant either for body decoration or as a medicine. Also use the stems and branches fumigants are used to treat rheumatic and back pains. Recent study on *T. brownii* wood and bark showed good activity to control plants pathogenic fungi (*Aspergillus niger, A. flavus, Natrassia mangifera* and *Fusarium moniliform*) (Salih and Hiba, 2010).

2.1.1.2 *Abrus precatorius* L

Abrus precatorius is known in Sudan as Habat Alarous. It belongs to family Fabaceae, a woody twinning plant with characteristic red and black seeds. The leaves are pinnate and glabrous, with many leaflets (12 or more) arranged in pairs. The leaflets are oblong, measuring 2.5 cm long and 1.5 cm wide. The plant bears orange-pink flowers, which occur as clusters in short racemes that are sometimes yellowish or reddish purple in color, small and typically pea like. The plant produces short and stout

brownish pods, which curl back on opening to reveal pendulous red and black seeds, 4 to 6 peas (Fig 2.1) in a pod (Ivan, 2003).

It grows in tropical climates such as India, Sri Lanka, Thailand, the Philippine Islands, South China, tropical Africa and the West Indies. It also grows in all tropical or subtropical areas. The most poisonous parts of the plant involved in poisoning are the small, scarlet seeds that have a black eye at the hilum. The seed contains the toxic poison abrin which is close relative to ricin. Ingested seeds can affect the gastrointestinal tract, the liver, spleen, kidney, and the lymphatic system. Infusion of seed extracts can cause eye damage after contact. The roots, stems, and leaves also contain glycyrrhizin (Windholz, 1983). The seeds were also used to treat diabetes and chronic nephritis. The plant is also used in some traditional medicine to treat scratches and sores, and wounds caused by dogs, cats and mice and are also used with other ingredients to treat leucoderma. They are ground with lime and applied on acne sores, boils, and abscesses. The plant is also traditionally used to treat tetanus, and to prevent rabies. Various African tribes use powdered seeds as oral contraceptives (Watt and Breyer-Brandwijk, 1962). Boiled seeds of *A. precatorius* are eaten in certain parts of India (Rajaram and Janardhanan, 1992). In Egypt seeds are taken orally with honey as an aphrodisiac (Salah et al., 1979). In Kenya fresh leaf juice is taken orally for coughs.

In Sudan it grows wildly in the southern part of Sudan. Seeds are traditionally used as and to treat diabetes and chronic nephritis, Hot water extract of the seed is taken orally as an antifertility agent (contraceptive) and in spiritual healing (Hussein and Baerheim-Suendsen, 1981).

Fig 2.1 *Abrus precatorius L*

2.1.1.3 *Combretum hartmannianum* Schweinf

The largest genus of the family Combretaceae is *Combretum*, comprising of about 250 species and distributed throughout the tropics and subtropics. *Combretum* is absent from Australia and the Pacific Islands (Wickens, 1973). *C. hartmannianum,* is widespread throughout the Sahel belt from Senegal to Cameroon, and eastwards to the Sudan. A shrub up to 4 m as a tree under favorable conditions 10 m high known locally in Sudan as (Habeel Al Gabal). Leaves alternate, shining light green when young, typically rust-colored when mature, leaves were used as an antipyretic, diuretic and for various diseases such as yellow fever, hepatic disorder (Maydell, 1990)

For different parts of plant such as root, stem, bark and fruit some medicinal applications are described for the treatment of influenza, rheumatism, intestinal, worms, coughs, colic, impotence, haemorrhoids, constipation, anorexia, malaria, wounds and syphilis. Research has shown that phenolic compounds like gallic tannins extractable by

methanolic solutions are responsible for some of the positive health effects (Pousset et al., 1993; Asres et al., 2001).

Elegami et al., (2002) have found in their screening of forty eight extracts of four members of Combretaceae that leaf, bark, fruit and stem bark extracts of both *C. pentagonum* Lawson and *C. hartmannianum* Schweinf gave good effects against gram-positive and gram negative bacteria, and most of the activity was found in water and methanol extracts, where as chloroform extracts were devoid of activity. Extracts made from different parts of the plants did not differ much from each other in antimicrobial activity. The species of *Combretum* included in this study were found to be rich in flavonoids, saponins and terpenes as well as tannins, why the authors speculated that the good antibacterial effects shown by these species might be due to tannins and flavonoids. The antibacterial effects of *C. hartmannianum* are in accordance with the use of this plant in traditional medicine in Sudan, where it is used for treatment of fever, jaundice and bacterial infections (El Ghazali et al., 1994; Al Magboul et al., 1988).

2.2 Material and Methods

2.2.1 General Strategy

The main purpose of this study is to discover anti-acne compounds from selected Sudanese medicinal plants. Initially, it was necessary to collect and identify the plant species. After that, all plants species were extracted. The phenolic, flavonoids and Tannin content were analyzed to promising extracts. The samples were evaluated for anti-acne potency based on bacterial, lipase inhibitor and anti-oxidant activity. The ones

resulting best anti-acne potency were further investigated to isolate and chemically characterize the substance responsible for this biological activities (Fig 2.2).

2.2.2 Collecting and Identification of plants

Forty plants species used in this study were collected from Khartoum (Shammbat, Oumdruman market, Abu Rouf -Nile river bank) and Al Qadarif states (near to Ethiopian border, about 410 kilometers from the capital) in Sudan (Fig 2.3). The specimens were Identification and authenticated by the Department of Plant Botany and Biotechnology, Faculty of Agriculture and faculty of forest, University of Khartoum. Voucher specimens are deposited in the Horticultural Laboratory, Department of Horticulture, Faculty of Agriculture, University of Khartoum (Table 2.1).

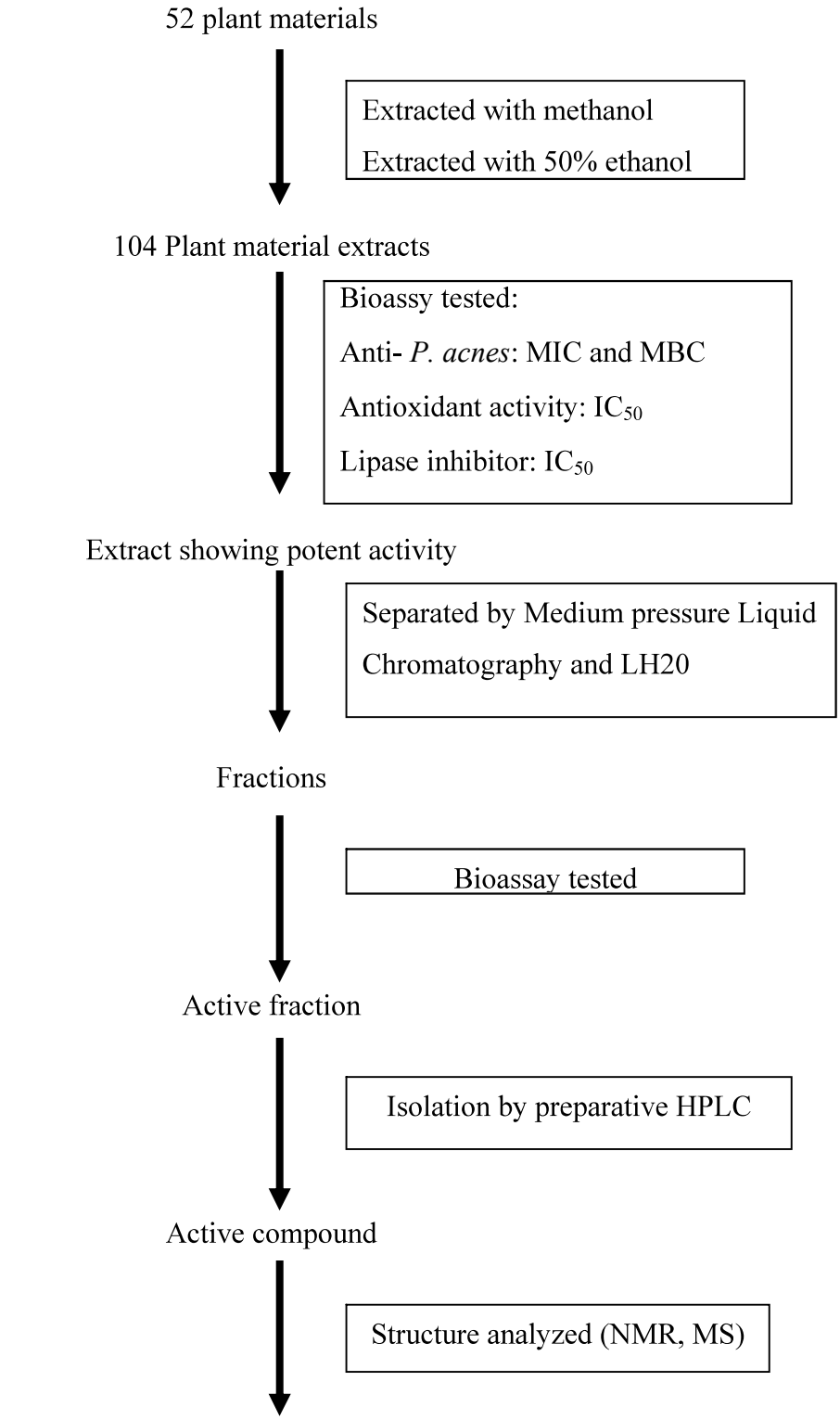


Fig 2.2. General methodology

Fig 2.3. Map of the Republic of Sudan

Plant collections were carried out in the Khartoum and Al Qadarif states.

Source: (http://commons.wikimedia.org/wiki/File:Map_of_Sudan_(New).jpg)

2.2.3 Extraction methods

Plant materials were shade dried at room temperature and powdered before being extracted with methanol and 50% (v/v) ethanol in water (ratio of 1 g sample: 10 ml solvent) for 12 h three times. The extracts were filtered and then the solvent was removed under vacuum using rotary evaporator in 30ºC. The concentrated extracts were then dried under freeze drying. The crude extracts in yields are listed in Table 2.1.

2.2.4 Reagents

Dimethyl sulfoxide (DMSO), GAM Broth Modified"Nissui", glucose, (+)-Catechin, yeast extract (Difco, France), nutrient broth (Difco, France), Folin Ciocalteu , Bovine Serum Albumin (BAS) and Tween-80 (MP Biomedicals LLC, France) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tetracycline hydrochloride was obtained from Sigma Chemical Co. Ltd (China). 2-(N-morpholino) ethanesulfonic acid (MES) and gallic acid were from Naka Lai Tesque, INC. (Kyoto, Japan). Isopropyl methylphenol (IPMP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and vanillin were from TCI (Tokyo, Japan). Lipase kits were from Pharma Biomedical, (Osaka, Japan).

2.2.5 Bioassay methods

2.2.5.1 Antimicrobial analysis

2.2.5.1.1 Test microorganism

Propionibacterium acnes ATCC 6919 was obtained from Biological Resource Center (NBRC), National Institute of Technology and Evolution, Chiba, Japan. The bacterial colonies were maintained in a medium consisting of GAM broth

modified“Nissui”0.5%, glucose 1.0%, yeast extract 0.3%, nutrient broth 0.5%, and 0.2% Tween-80 .

2.2.5.1.2 Evaluation of antibacterial activity

The broth dilution method was used to determine the minimum inhibitory concentration (MIC) of each plant extract. This assay was determined as described by Chomnawang et al., (2005). Briefly one hundred microliters of each extract were two-fold serially diluted with 10% DMSO, 95 µL of sterilized medium and inoculum 5 µl were added to each well of a 96-well plate. The inoculum was prepared at the density of 1×10^6 CFU/ml approximately. The broth culture was incubated for 72 h under anaerobic conditions. Extract concentrations at which there was no visually detectable bacterial growth was described as the MIC.

Ten µl of each media with no visually detectable bacterial growth were inoculated in 100 µl media. The concentration at which there was no bacterial growth after second inoculation was described as MBC (Minimum Bactericidal Concentration). The negative control used was DMSO while the Tetracycline hydrochloride and isopropyl methylphenol (IPMP) were used as a positive control. Each experiment was carried out in triplicate (Batubara et al., 2009).

2.2.5.2 Lipase inhibitory analysis

P. acnes was cultured in media (the same as the media in antibacterial assay). The cell suspension was centrifuged at 900 g for 10 min at 4ºC. The precipitate was diluted in phosphate buffer saline (PBS) at pH 6.98. The bacteria in this solution were destroyed by micro homogenizing system (TOMY Micro Smash MS-100) at 4000 rpm for 30 s and centrifuged at 5000 g for 60 s. The filtrate was collected and placed in a

dialysis tube for 6 days. The crude enzyme was lyophilized in freeze drier to be a powder form and was used for successive experiments.

Lipase inhibitory activity assay was conducted using the dimercapto propanol tributyrate (BALB) method (Furukawa et al., 1982). The reagents were 390 µl of 5,5' – dithiobis (2- nitrobenzoic acid) (DTNB) in Tris buffer (coloring agent), 10 µl of phenyl methyl sulphonyl fluoride (PMSF, esterase inhibitor), 200 µl lipase and 25 µl of sample or solvent (DMSO). All reagents (Dainippon Sumitomo Pharma CO, Ltd, Japan) were added to two tubes (1 and 2) and were both incubated at 30ºC for 5 minutes. Fifty µl of BALB solution (substrate) was added to tube 1 and the solutions in both tube were mixed well and incubated at 30ºC for 30 minutes. After 30 minutes, the reaction in the tubes was stopped by adding 490 µl of stopping reagent. After addition of stopping reagent, a 50 µl of BALB solution was added in tube 2 after which the solutions in both tubes were mixed well and centrifuged to remove the insoluble materials. The absorbance of each tube was measured at 414 nm. Each experiment was performed in triplicate. Principle reaction is shown in Fig (2.4).

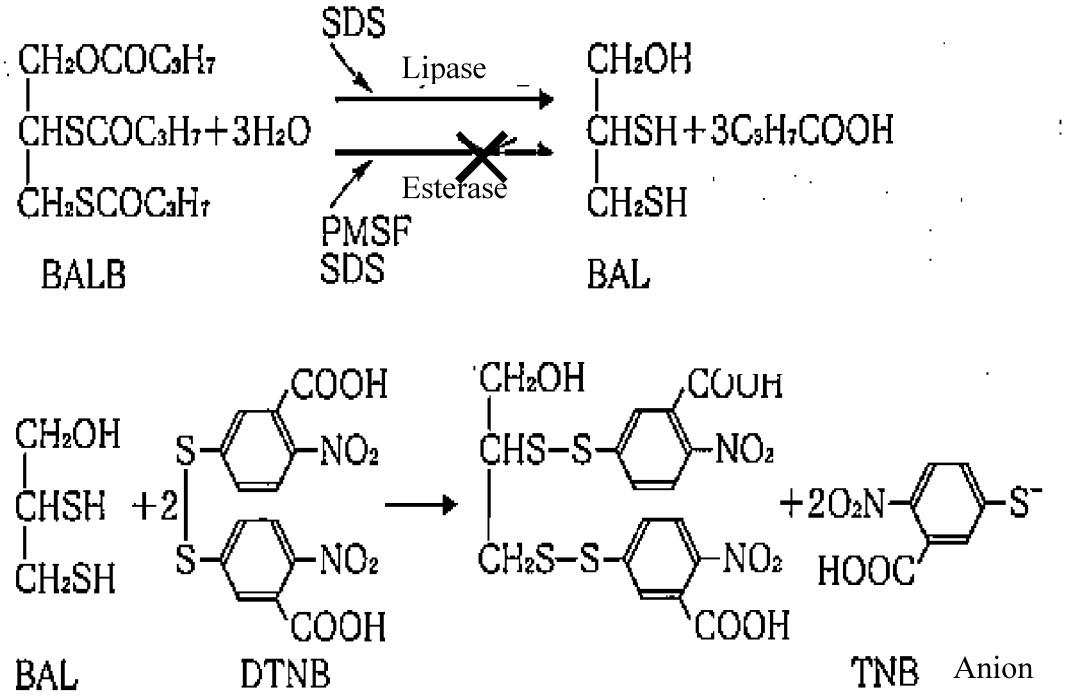


Fig 2.4. Reaction between BALB and DTNB

Lipase inhibition was calculated as:

% inhibition= [(A solvent – A sample)] /A solvent X 100%

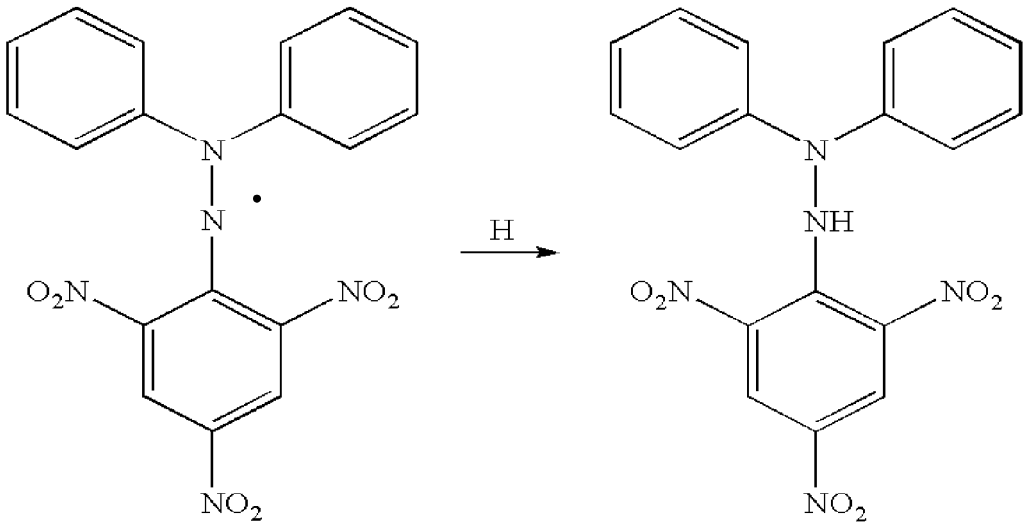
Where A solvent was the difference between absorbance of tube 1 and tube 2 in solvent (DMSO) and A sample was the difference between absorbance of tube 1 and tube 2 of sample. Tetracycline hydrochloride and isopropyl methylphenol (IPMP) were used as a positive control (Batubara et al., 2009)

2.2.5.3 Antioxidant analysis

The antioxidant assay used in this study adopted a free radical- scavenging activity using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) test. The molecule of DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet color, characterized by an absorption band in ethanol solution centred at about 520 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color (although there would be expected to be a residual pale yellow color from the picryl group still present). Samples were diluted in the ethanol to make a final concentration of 1.67, 3.33, 6.67, 10.00, 13.33, 16.67, 33.33, 66.67, 100.00, 133.33, 166.67 µg/ml. A 100 µl of sample, 100 µl of MES (2(N-morpholino (ethane sulfonic acid) buffer pH7.4 and 100 µl of DPPH (11.8 mg DPPH in 100 ml ethanol) were added to each well of a 96-well plate. After 30 minutes, the absorbance of the mixture was measured at 510 nm. The reaction in DPPH is shown in Fig (2.5). The positive control was (+) catechin, while ethanol was used as the blank. The inhibitory activity was calculated according to the following equation:

% inhibition = 1- [(A sample – A control) / (A blank – A control)] X 100%

Where A sample was the absorbance of sample, A control was the absorbance of (+) catechin and A blank was the absorbance of ethanol. Each sample concentration and positive control was tested in triplicate. (Batubara et al., 2009)



Free radical Non radical

Fig 2.5. Schematic representation of chemically stable radical scavenging activity using DPPH as stable free radical (Blios, 1958)

2.2.6 Chemical analysis

2.2.6.1 Determination of total phenolic

The total phenolic assay was performed as described previously by Ainsworth and Gillespie (2007). Plant extracts were dissolved in 50% methanol and one hundred microlitres were transferred into test tubes, followed by 200 µl 1N Folin Ciocalteu reagent 10% (v/v). It was then mixed with 800µl of sodium carbonate (700 mM) and maintained at room temperature for 2 h. Two hundred microlitres of samples from the assay tube was transfer to 96-well microplate and read the absorbance at 765 nm using microplate reader. Total phenolic concentrations were expressed as gallic acid (GAE) equivalents. Data are expressed as milligram of gallic acid equivalents.

2.2.6.2 Vanillin assay for flavanoids

This assay was performed as described previously by Hagerman (2002). Plant extracts (50 µl) (in triplicate) were made up to 1 ml with methanol in test tubes before adding 2.5 ml methanolic–HCl (95:5, v/v) and 2.5 ml of 1% vanillin reagent in methanol. Similar preparations of a blank that contained methanol instead of plant extracts were made. After 20 min at 30 °C, absorbance at 500 nm was read using a UV–visible spectrophotometer (JASCO V-520, Japan). The flavonoids in the plant extracts were expressed as catechin equivalents

2.2.6.3 BSA assay for Tannin

Tannin content of plant extracts was determined by a protein precipitation method using Bovine Serum Albumin (BSA). This method was described by Batubara et al. (2012) by analyzing the BSA content of the supernatant liquid. Samples were diluted in 50% (v/v) ethanol/water to prepare 2 mg/ml of concentration. A 200 ul volume of sample solution was added to 200 µl of BSA solution (10mg/ml, dissolved with 0.1 M acetate buffer, pH 5). After reaction at room temperature for 1h, the solution was centrifuged at 13000g for 2 min. The remaining BSA in the supernatant was determined by HPLC with a reversed phase Develosil300C4-HG-5 column (4.6i.d.X150mm, Nomura Chemical Co, Ltd, Japan) monitored at 280 nm. The solvent system used was as follow; a linear gradient elution for 20min from 80 to 20% solvent A (0.01% TFA in water) in solvent B (90% (v/v) CH3CN/water containing 0.01% TFA) at flow rate 1 mL/min. The column temperature was 35°C.

2.3 Results and Discussion

In the present work, screening program start with the 104 extracts belonging to 40 plant species distributed among 28 families. The anti-acne activity of some Sudanese medicinal plant extracts were determined by using an antibacterial assay, lipase inhibitory activity and a DPPH radical scavenging assay. Table (2.1) summarizes the scientific name, family name, part used and voucher specimen of the medicinal plants. Within these selected Sudanese medicinal plants *Lawsonia inermis* L., *C. hartmannianum*, *Acacia seyal* var fistula and *Solanum dubium* are used for preventing the dryness and bacterial infection of the skin, other plants are used as anti-inflammation, anti-malaria, anti-diarrhea, antimicrobial infection and other diseases (El Ghazali et al., 1994).

Generally polar solvents such as methanol, ethanol, either and their aqueous mixtures, are mostly recommended for the extraction of phenoilcs from the plants matrix. For example, in several studies pure and aqueous mixtures of methanol and ethanol have been used to extract compounds with antioxidant properties from different plant materials including fruits, vegetable grains, medicinal plants and agro-wastes (Chatha et al., 2006; Shabbir et al., 2011). In this study we used methanol and 50% ethanol as solvent for plant extraction. The yield of extracts is has tendency that the yield of methanol extracts is higher than the ethanol 50% extract. The highest yield of extracts were found on *Balanites aegyptiaca* fruits and *Acacia nilotica* (L.) pods methanol extracts while the lowest yield were found on *T. brownii* and *C. hartmannianum* wood 50% ethanol extracts.

2.3.1 The antimicrobial activity

The antibacterial effects of Sudanese medicinal plants against acne causing bacteria have been rarely addressed, even though attention has been given to this pathogen in other geographical ethnobotanical-relevant studies (Chomnawang et al., 2005, Tsai et al., 2010; Balakrishnan et al., 2011). The antibacterial MIC values of the extracts of 40 medicinal plants species used are presented in Table (2.1). Gibbson (2005) suggested that isolated phytochemicals should have MIC< 1 mg/ml, so that in this study MIC value 0.13 mg/ml was considered to be an indication of excellent antibacterial activity. Among the extracts used in this study the species belong to Combretaceae family comparatively showed a good antibacterial activity, specifically all extracts from *T. laxiflora* showed the best antibacterial activity against *p. acne* (MIC 0.13 mg/ml), followed by methanol extract of *T. brownii* (Bark), methanol and 50% ethanol of *C. hartmannianum* (wood) extracts exhibited a medium antibacterial activity (MIC 0.5 mg/ml). According to Elegami et al., (2002) water and methanol extracts of leave, fruit and stem bark from *C. hartmannianum*, exhibited an activity against gram-positive bacteria which due to flavonoids, saponins, terpenes and tannins. Antimicrobial investigation of some species of *Terminalia* and *Combretum* which belong to Combretaceae indicated that the most effective extracts were the methanol extracts of roots of *T. sambesiaca*, *T. kaiserana*, *T. sericea*, *C. fragrans* and *C. padoides*. All these extracts showed a remarkable growth inhibition against Gram-positive bacteria (Fyhrqist et al., 2002). Although the activity of *T. arjuna* (Bark) against acne bacteria is known (Vijayalakshmi et al., 2011) potency of *T. laxiflora* (wood) against *P. acnes* has not yet been investigated.

2.3.2 The antioxidant activity

DPPH is a stable radical that is used as a popular method of screening for free radical scavenging ability, antioxidant activity in particular of plant extracts (Sharma and Bhat, 2009). This assay was used to evaluate the free radical scavenging activity of our plant extracts. The result in Table (2.1) showed that ten plant extracts have partially good potent activity with less than 5 μg/ml of IC_{50}, comparing with positive control of (+)-catechin (IC_{50} 2.39 μg/ml). In addition methanol extract of *Acacia nilotica* (pods) demonstrated the lowest IC_{50} for scavenging activity of the DPPH radical (IC_{50} 1.32 μg/ml) and this could be due to some isolated compounds from this plant, such as galloylated catechins and gallocatechin derivatives (Maldini et al., 2011). Also Singh et al., (2010) has mentioned that the strong antioxidant activity of *A. nilotica* (bark and leaves) was due to umbelliferone.

2.3.3 Lipase inhibitory activity

Results of the lipase inhibitory activity shown in Table (2.1) clarified that the methanol extract of *A. nilotica* (pod), 50% ethanol extracts of *A. precatorius* (seed) and *T. laxiflora* exhibited the strongest inhibitory effect on lipase with more than 70% inhibition. These values were higher than those of the positive controls such as tetracycline hydrochloride and IPMP at the same concentration. Methanolic extract of *T. laxiflora* and *A. nilotica* bark showed the second strongest activity with an inhibition of 69.5% and 66.0% respectively. Seven plant extracts expressed moderately strong activity with more than 50% inhibition. Batubara et al., (2009) reported that some extract from Indonesian medicinal plants had better activities than the positive controls (chloramphenicol, tetracycline, and IPMP). A study done by Falcocchio et al., (2006) indicated that (+)-catechin and kaempferol are promising candidates for treatment of

acne due to their strong inhibitory activity on *P. acnes* lipase GehA (glycerol-ester hydrolase A), as well as to their wide anti-acne properties and their low toxicity. Previously phytochemical studies of *A. nilotica* resulted in the identification of a variety of phenolic constituents, among which kaempferol was identified (Singh et al., 2008). The best extract based on comprehensive activities was *T. laxiflora* 50% ethanol extract with better MIC value 0.13 mg/ml, antioxidant activity (IC_{50} 3.45μg/ml) and lipase inhibitory activity (74.1%).

Table 2.1. Anti-acne properties of selected Sudanese medicinal plant extracts

No.	Scientific name	Family	Used part	Voucher Specimen	Extract	Yield (%)[a]	MIC mg/ml	Lipase Inhibition (%)	Antioxidant IC_{50}(µg/ml)
1	*Parkinsonia aculeata* L.	Fabaceae	Leaves	SD-SH-02	M	43.2	-[b]	24.63±0.18[c]	nd
2					E	8.12	-	21.67±0.09	46.71±0.14[c]
3	*Abrus precatorius* L.		Seeds	SD-OD-22	M	16.8	-	51.59±0.92	7.97±0.29
4					E	6.80	2.00	76.79±0.80	8.631±0.05
5	*Trigonella foenum-graecum* L.		Seeds	SD-OD-44	M	17.9	-	nc	nd
6					E	8.55	-	27.75±0.11	23.37±10.71
7	*Ambrosia maritima* L.	Asteraceae	Arial parts	SD-SH-03	M	16.3	2.00	36.9±0.03	30.79±0.40
8					E	4.18	4.00	28.48±0.11	40.56±0.60
9	*Vernonia amygdalina* Del.		Leaves	SD-MAPI-19	M	31.1	-	21.07±0.32	nd
10					E	13.4	4	12.57±0.13	21.67±2.84
11	*Aristolochia bracteolata* LAM	Aristolochiaceae	Arial parts	SD-SH-04	M	36.8	-	15.23±0.21	nd

12					E	11.8	4	6.04±1.69	21.67±2.84
13	*Citrullus coloynthis* (L.) Schrad	Cucurbitaceae	Dry Fruit	SD-OD-05	M	34.5	4.00	1.11±0.45	nd
14					E	12.0	4.00	6.91±0.15	nd
15	*Ziziphus spina –christi* (L.) Desf.	Rhamnaceae	Fruits	SD-SH-06	M	48.8	-	nc	nd
16					E	6.89	4.00	2.21±0.52	33.13±2.27
17			Bark		M	36.8	2.00	53.15±0.44	3.62±0.56
18					E	5.54	-	35.40±1.21	6.22±0.70
19			Leaves		M	47.1	2.00	14.94±0.24	24.57±0.44
20					E	9.23	-	nc	21.88±0.57
21	*Lawsonia inermis* L.	Lythraceae	Leaves	SD-SH-07	M	39.6	1.00	22.20±0.09	11.87±1.37
22					E	6.20	4.00	11.62±0.19	15.36±1.20
23	*Moringa oleifera* lam.	Moringaceae	Leaves	SD-SH-08	M	20.3	-	18.08±0.06	50.25±4.57
24					E	12.0	-	27.34±0.07	18.47±0.52
25	*Salvadora persica* L.	Salvadoraceae	Leaves	SD-SH-09	M	36.5	1.00	3.16±0.18	nd
26					E	5.76	2.00	12.03±0.18	24.50±5.62
27			Stems		M	18.4	-	5.83±0.95	71.61±0.63
28					E	5.17	4.00	1.47±1.25	nd
29	*Tamarix nilotica*	Tamaricaceae	Stems	SD-OD-10	M	40.5	4.00	34.49±0.30	10.92±0.63

	(Ehrenb.)Bunge								
30					E	12.2	-	3.84±0.19	8.30±0.43
31	*Calotropis procera*	Asclepiadaceae	leaves	SD-SH-11	M	26.9	-	15.36±0.26	nd
32					E	14.0	-	13.18±0.03	nd
33	*Solenostemma argel* Hayne		leaves	SD-SH-23	M	37.2	-	6.34±0.05	nd
34					E	13.5	-	16.31±0.18	24.37±3.41
35	*Xanthium brasilicum* W.	Compositae	leaves	SD-SH-12	M	23.4	4.00	16.73±0.08	24.71±4.44
36					E	9.7	-	8.27±0.04	24.38±4.25
37	*Lepidium sativum* L.	Cruciferae	seeds	SD-MAPI-13	M	37.4	4	5.021±0.21	nd
38					E	3.69	-	4.55±0.20	33.26±3.79
39	*Ammi visnaga L.*	Umbelliferae	Fruits	SD-OD-38	M	20.0	4	nc	nd
40					E	13.8	4	8.17±0.17	nd
41	*Khaya senegalensis* (Desr.) A. Juss.	Meliaceae	Bark	SD-SH-14	M	62.1	-	20.12±0.76	3.37±0.68
42					E	5.75	4.00	38.73±0.28	12.20±1.17
43	*Balanites aegyptiaca* (L).Del.	Balanitaceae	leaves	SD-SH-15	M	25.5	-	12.28±0.29	nd
44					E	19.0	-	nc	nd
45			Fruit		M	74.4	4.00	nc	nd

46					E	6.45	4.00	6.91±0.07	nd
47			Bark		M	40.7	-	nc	nd
48					E	3.61	-	nc	nd
49			Wood		M	5.64	4.00	25.06±0.47	nd
50					E	3.15	2.00	2.39±0.10	nd
51	*Carum carvi* L.	Apiaceae	Fruits	SD-OD-47	M	29.4	-	nc	nd
52					E	12.58	-	10.74±0.25	31.76±1.27
53	*Hibiscus sabdariffa* L.	Malvaceae	Flowers	SD-SH-48	M	58.4	4.00	3.64±0.04	22.14±0.64
54					E	27.48	4.00	16.15±0.19	68.13±4.25
55	*Abutilon pannosum* (Forst.f.)Schlecht.		Leaves	SD-SH-43	M	16.1	2.00	2.43±0.23	nd
56					E	9.45	4.00	17.46±0.05	92.098
57	*Terminalia brownii* Fres.	Combretaceae	Bark	SD-GF-02	M	43.0	0.50	51.67±3.62	3.85±0.37
58					E	4.46	2.00	59.91±1.76	5.89±0.09
59			Wood		M	12.5	4.00	15.53±0.98	4.97±1.69
60					E	1.85	4.00	54.30±0.26	5.19±0.93
61	*Combretum hartmannianum* (Schweinf.)		Bark	SD-KH-04	M	21.9	1.00	45.76±0.69	4.99±0.44
62					E	4.88	2.00	48.34±0.79	6.7±0.33

63			Wood		M	4.68	0.50	10.19±0.14	9.74±0.67
64					E	1.86	0.50	40.45±0.07	8.21±0.25
65	*Terminalia laxiflora* Engl& Diels		Wood	SD-KH-03	M	14.3	0.13	69.51±0.45	4.44±0.13
66					E	2.04	0.13	74.14±0.17	3.45±1.33
67	*Guiera senegalensis* J.F.Gmel		Leaves	SD-OD-40	M	26.2	-	25.78±0.79	6.30±0.59
68					E	12.7	4.00	42.27±1.50	10.93±0.42
69	*Acacia seyal* var seyal Del.	Mimosaceae	Bark	SD-GF-05	M	32.9	2.00	42.33±3.47	4.03±0.51
70					E	6.44	4.00	33.23±1.92	6.96±0.66
71			Wood		M	2.52	-	3.01±1.13	8.87±0.93
72					E	2.17	-	20.21±0.11	9.71±1.79
73	*Acacia seyal* var fistula (Schweinf.)		Bark	SD-GF-06	M	27.1	-	25.22±2.21	7.53±0.11
74					E	6.88	-	1.31±0.09	10.28±1.23
75			Wood		M	8.54	4.00	nc	nd
76					E	2.74	-	19.24±0.01	nd
77	*Acacia tortilis* (Forssk.) Hayne		Bark	SD-KH-07	M	5.70	4.00	nc	15.01±0.04
78					E	2.30	4.00	7.75±1.36	27.39±1.82

79			Wood		M	5.70	4.00	1.41±2.63	9.87±0.73
80					E	6.53	4.00	21.29±0.19	nd
81	*Acacia nilotica* (L.)		Pods	SD-OD-01	M	70.5	4.00	75.35±0.58	1.32±0.97
82					E	6.15	2.00	53.09±1.12	8.11±0.64
83			Bark		M	23.75	-	66.53±0.97	3.34±0.33
84					E	5.42	1.00	53.84±0.35	3.20±0.68
85	*Haplophyllum tuberculatum* Forsk	Rutaceae	Aerial part	SD-WR-32	M	17.2	-	nc	nd
86					E	8.75	4	1.72±0.10	109.3±5.22
87	*Kigelia africana* (lam.)Benth.	Bignoniaceae	Fruits	SD-SH-21	M	26.2	-	nc	24.99±1.25
88					E	8.65	1.00	15.22±0.24	37.34±6.47
89	*Hyphaene thebaica* (L.)Mart.	Palmae	Fruits	SD-OD-41	M	52.9	4.00	0.67±0.02	nd
90					E	14.4	4.00	7.95±1.39	18.42±0.20
91	*Moringa oblongifolia* (Frosk.)A.Rich	Capparidaceae	Stems	SD-SH-45	M	10.7	4.00	nc	nd
92					E	4.20	4.00	6.46±0.17	20.79±2.30
93	*Capparis deciduas* (Forsk) Edgew.		Stems	SD-SH-17	M	15.9	-	nc	nd
94					E	4.13	4.00	nc	nd

95	*polygonum glabrum* Willd.	Polygonaceae	Leaves	SD-SH-A-03	M	33.7	4.00	30.79±0.55	6.88±0.16
96					E	8.18	4.00	31.02±0.54	5.170±0.38
97	*Fagonia cretica L.*	Zygophyllaceae	Aerial part	SD-SH-26	M	14.7	-	1.00±0.18	nd
98					E	8.30	1	0.18±0.14	nd
99	*Nigella sativa* L.	Ranunculaceae	Seeds	SD-OD-16	M	19.3	-	0.43±0.09	44.49±0.69
100					E	7.00	-	5.18±1.93	nd
101	*Solanum dubium* Fresen	Solanaceae	Fruits	SD-SH-34	M	37.2	-	2.61±0.11	10.66±0.41
102					E	10.9	4.00	7.36±0.10	13.77±2.53
103	*Grewia tenax* (Forsk)	Tiliaceae	Fruits	SD-OD-42	M	69.9	-	nc	nd
104					E	8.46	-	3.58±0.12	183.6±31.81
	IPMP						1.00	48.28±0.41	*
	Tetracycline [e]						0.03	64.06±0.26	*
	(+)-Catechin						*	*	2.39±0.97

MIC, Minimum inhibitory concentration; [a] Based on dried weight, [b] No inhibitory activity at concentration of 4.00 mg/ml, [c] Data given as mean ± standard deviation of triplicate test; nc, inhibition could not be calculated because there is no activity at the highest concentration of 500 µg/ml; [e] MIC value in µg/ml; nd, Failed to achieve 50% inhibition at highest concentration 166.7 µg/ml; * Not tested; M, Methanol extract; E, 50% Ethanol extract

2.3.4 Tannin, Flavonoid and phenolic content

The chemical analysis was performed to find the perspective chemical group which responsible for biological activity. Total phenolic and flavanoid contents of active extracts were determined spectrophotometerically, while total tannin content was determined by the ability of BSA precipitation, which refer to condensed and hydrolysable tannins presence in the extracts. Vanillin HCl method is widely used to estimate the proanthocyanidin in the extracts. The result for total phenolic, flavanoid and tannin contents of selected extracts are presented in Table (2.2) Almost all selected extract contained phenolic ranged from 34.9-47.9 μg/mg. Total Phenolic content previously reported had strong relationship with antioxidant activity (Piluzza and Bullitta, 2011). Many useful properties of flavanoids have been reported including anti-inflammatory activity, enzyme inhibition and antimicrobial activity (Havsteen, 1983) .The highest amounts of flavanoids (38.87 μg/mg) were detected in methanol extract of *T. brownii* bark and the lowest amount (0.14 μg/mg) was detected in methanol extract of *T. laxiflora* wood.

Tannins exhibit many biological activities. In previous studies, tannins have been evaluated as antibacterial, antiviral, radical scavenging, antitumor activities and inhibitory activities for some enzymes (Bruyne et al. 2009; Okuda, 2005). No tannin content was detected in *A. precatorius* fruit. Extract of *A. nilotica* (L.) bark, *T. brownii* bark and *T. laxiflora* wood (methanol, ethanol 50%) have partially high percentage of total tannin (88.01, 82.83, 81.91 and 73.3%) respectively. Among them the extracts of *T. laxiflora* wood have less or no flavanoids, these result indicate that hydrolysable tannins may included in these extracts .while condensed tannin would be included in *A. nilotica* (L.) and *T. brownii* barks.

2.4 Conclusion

These findings demonstrated that the study of medicinal plants from Sudan confirmed a promising inhibitory effect in anti-acne. Toxicity and pharmacological studies are also needed to support the safety of these plants for cosmetic uses. Fractionation, bioassay-guided isolation and chemical characterization (structure elucidation) of some of the anti-acne potent compounds from crude extract of *T. laxiflora* will describe in next chapter.

Table 2.2. Total phenolic, flavanoid and total tannin contents of selected plant extracts as anti-acne activities

Sample name	Parts used	Extract	Total phenolic [a](µgGAE/mg)	Flavanoids [b](µgCAE/mg)	Total tannins [c] (% BSA)
A. precatorius	Seeds	E	34.98±2.83	nd	nd
T. brownii	Bark	M	47.89±4.99	38.87±1.20	82.83±10.38
C. hartmannianum	Wood	M	36.01±1.77	nd	28.70±20.54
		E	35.49±3.31	nd	31.73±21.62
T. laxiflora	Wood	M	41.09±3.74	0.14±0.01	81.91±9.921
		E	36.72±1.63	nd	73.31±17.79
A. nilotica	Pods	M	44.40±4.15	18.55±0.27	53.68±9.30
	Bark	M	35.73±4.83	26.17±0.55	88.01±9.42

a Values expressed as gallic acid equivalent (GAE) per mg of plant extract

b Values expressed as catechin equivalent (CAE) per mg of plant extract

c Values expressed as percentage of BSA precipitated per mg of plant extract

nd not detected

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Chapter 3
Anti-acne activity of tannin related compounds isolated from *Terminalia laxiflora* Engl & Diels

3.1 Introduction

Acne vulgaris is the most common skin disease that affects areas containing the largest oil glands including face, back and trunk (Park et al., 2004). *P. acnes* have been implicated over other cutaneous microflora in contributing to the inflammatory response of acne. It acts as an immunostimulator through producing enzymes such as lipases and proteases which are involved in the development of inflammatory process (Toyoda and Morohashi, 2001). Several treatments have been introduced to decrease the aesthetic and psychological problems caused by acne. The topical application of therapeutic agents has been found to be more feasible than hormonal treatment and laser therapy. The ingredients in topical acne treatments, particularly herbs and naturally derived compounds, have received considerable interest as they show fewer adverse effects than synthetic agents (Kanlayavattanakul and Lourith, 2011).

It has been estimated that less than 1 – 10 % of the large diversity of 250.000 – 500.000 plant species on the Earth have been studied chemically and pharmacologically for their medicinal properties (Farnsworth and Soejarto, 1991; Verpoorte, 2000). This is especially true for the tropical flora, as at date only 1 % of the species in these habitats have been studied for their pharmaceutical potential (Gurib-Fakim, 2006). Tropical forests and many other tropical ecosystems are rich sources of a diversity of plant derived chemical compounds, both because of the high species diversity but also because of the "eternal summer" which forces the plant species to the constant production of chemical defense compounds against herbivores and pathogens as well as against other plant species. Plants in a tropical rainforest also have to compete for space and light and this forces species to develop more efficient means of using energy and nutrients as well as to allocating resources for secondary compound production. For

these reasons a greater portion of the tropical plant species contains secondary compounds, potentially useful as models for/as medicines (Wood-Sheldon et al., 1997). Plant derived compounds have been, and are still, important as such or as models (lead compounds) for medicines: 50 % of the prescription products in various countries in Europe and the US are either natural products or natural product derivates (Newman et al., 2003). To date about 50 drugs have come from tropical plants (Gurib- Fakim, 2006).

Based on our previous screening experiments for anti-acne activity, the methanolic and 50% ethanolic extracts of *Terminalia laxiflora* Engl & Diels wood showed potent activity among the 40 species of Sudanese medicinal plants examined .

3.1.1 The genus Terminalia L.

The second largest genus of Combretaceae family, *Terminalia*, consists of 200 species, distributed in the tropics and subtropics (Wickens, 1973; Tan et al., 2002). About 30 species of *Terminalia* are found in Africa (Wickens, 1973). Species of *Terminalia* vary greatly in morphology, anatomy and karyotype evidence (Stace, 1965; Ohri, 1996). The species of *Terminalia* are small to large trees depending on the growth habitat. In rainforests they can reach heights of up to 50 m, and often grow as emergents, reaching above the upper tree stratum. In Africa *Terminalia* species growing in Miombo woodlands are smaller, and usually reach heights of 10-20 m. The African species of *Terminalia* are most often trees and rarely shrublike in growth form. *Terminalia* trees show a very distinct, pagoda-like tree architecture, known as Aubréville`s Model: the main stem produces whorls of horizontal lateral branches and each lateral branch is made up of a succession of branchlet units, each with the tip turned up and a cluster of leaves at its apex (van Wyk and van Wyk 1997). Species of *Terminalia* lack scales or microscopic glands. In contrast to the Asian species of *Terminalia* with fleshy, hard,

resinous, 5- winged (-ridged) fruits, the fruits of the African species are usually 2-winged, papery and quite thin and very variable in shape and size, usually 1-3 cm long and 0.5-1.8 cm wide. The leaves of *Terminalia* species are usually spirally arranged, often crowded at the ends of the branches, sometimes on short shoots, rarely opposite, petiolate or subsessile. Two glands are often present at the base of the lamina or on the petiole. There are usually both hermaphroditic and male flowers in the same inflorescence. Usually the flowers are borne on axillary spikes with male flowers towards the apex and hermaphroditic flowers towards the base. Male flowers are stalked, the stalk resembling pedicels but corresponding to the lower receptacle with abortion of the ovary. Hermaphroditic flowers are sessile. The receptacle is divided into a lower part (lower receptacle) and an upper part, often scarcely developed, expanding into a shallow cup terminating into sepals. Petals are absent. Stamens are usually ten. The ovary is completely inferior; style free, not expanded at the apex. The flowers of *Terminalia* are remarkably uniform throughout the genus and scarcely ever provide any taxonomically useful characters and great reliance must therefore be placed on leaf, bark and fruit characters for species identification. Most of the African species of *Terminalia* are growing in various kinds of woodlands such as coastal woodlands and wooded grasslands, and some are typical components in riverine forests and rain forests (Wickens, 1973).

3.1.2 Traditional medicinal uses of some species of Terminalia, reported in the literature

About 200 woody species of *Terminalia* are used as resources in the timber, pharmaceutical, and leather industries (Srivastav, 1993). Although traditional healers throughout Africa have used species of the Combretaceae for the treatment of a wide range of disorders, only a few species have been subjected to scientific studies (Rogers

and Verotta, 1996). A number of studies have been carried out on the ethnomedical uses of *Terminalia* species in Africa. Many *Terminalia* species have various applications in African traditional medicine, such as for the treatment of hypertension, diarrhea, bacterial and fungal infections and fever, just to mention some of them (Hedberg et al., 1982; Fyhrquist et al., 2002). In Africa, all parts of the *Terminalia* species are used for medicinal purposes (Watt and Breyer-Brandwijk, 1962; Neuwinger, 2000), the fruits being reported to be used in only one case (Eldeen et al., 2005). In herbal remedies species of *Terminalia* are mostly used as hot water decoctions (woody plant parts) or infusions (leaves), but it is also common to mix dried, powdered plant parts with *Ugali*, maize porridge, and in some cases the fresh leaf sap is used. Sometimes decoctions of species of *Terminalia* are mixed with rice or maize porridge. Some of the species of *Terminalia* are used topically as well, mainly for the treatment of wounds, and are then applied as dressings or ointments. Sometimes the fumes of hot fomentations of the roots are inhaled for treatment of chest pains, presumably pneumonia or bronchitis.

According to Hartwell (1982) at least seven different species of *Terminalia* are used for the treatment of cancer in systems of Asian traditional medicine. Especially the fruits of the Asian species are frequently used for medical applications such as fever, cough, diarrhea, dysentery and skin diseases (Valsaraj et al., 1997) and even for food (Barthakur et al., 1991). There are several investigations, most of them of quite recent date, on the antibacterial and antifungal effects of both African, Asian and South American species of *Terminalia*. The antimicrobial effects have been evaluated with diverse methods, such as agar diffusion, agar dilution, direct bioautography and liquid serial dilution methods (microplates). In most of the investigations crude extracts have been evaluated for their antimicrobial effects, but in some cases active fractions and

compounds have been isolated and even evaluated for their antimicrobial potential. Some of the results on the antimicrobial effects of *Terminalia* species support their use in traditional medicine for treatment of infectious diseases. The genus *Terminalia* seems to include species with potent antimicrobial effects and good effects have been obtained against both gram-positive and gram-negative bacteria as well as against yeasts (*Candida* spp.), the basidiomycet *Cryptococcus neoformans* and dermatophytes (*Trichophyton* spp., *Epidermophyton* spp. and *Microsporum* spp.). Antimicrobial compounds have been found from all plant parts of *Terminalia* species. Several biological activities of *Terminalia sp*. have been reported such anti-inflammatory (Eldeen et al., 2006) diabetic (Kameswara et al., 2003), hypocholesterolemic (Rathore et al., 2004), anti-ulcer activity (Gupta et al., 2005), anticaries agent (Jagtap and Karkera, 1999), anti-HIV-1, anti-malarial (Valsaraj et al., 1997), antioxidant and melanin inhibitory activity (Manosroi et al., 2010).

Terminalia species contain an array of different compounds with biological effects such as hydrolysable tannins (punicalagin, terchebulin, isoterchebulin, chebulagic acid, chebulinic acid, mostly ellagitannins) and their monomers gallic acid and ellagic acid, methylated ellagic, acid derivatives, ethyl gallate, flavonoids, stilbenes, saponins, lignans, coumarins and triterpenoids. Many of the investigations on the biologically assay of isolated compounds from *Terminalia* spp. lack studies on the toxicity of the compounds, and these studies would be essential to predict the safety of use. Some organs of some species of *Terminalia* are reported to be poisonous, such as the roots of *T. sericea* (Watt and Breyer-Brandwijk, 1962) and thus care should be taken to prescribe the correct dosages. The use of extracts and especially of unstandardized extracts made of plants from the wild is not free of risks since the composition of the

secondary compounds may show great intraspecific variation. Therefore standardized extracts should be used if possible (Fyhrquist, 2007).

3.1.3 *Terminalia laxiflora* Engl & Diels

Terminalia laxiflora (local name Subagh – Darut) belongs to the family Combretaceae and is widely distributed in Africa, Middle and South East Asia. The Africa's distribution cuts across the savannah region, from the eastern Sudan to the western Senegal, and the northern Tunisia to the central Congo. It is a common indigenous tree in the woodland and semi - arid savannah of Sudan with a high potential of timber production and traditional medicinal uses (Kerharo and Adam, 1974).

The tree is of 12 m in height and nearly 1m width with the usual crooked bole, dark grey, deeply fissured and scaly bark (Kerharo and Adam, 1974). The wood is fire resistant because of its thick corky bark (Savill and Fox, 1967). The plant has a variety of medicinal applications across the areas of occurrence. These include the stem as chewing stick in Nigeria, and as gastric stimulant to prevent and cure diarrhoea in infants and children. It also aids digestion and relieves constipation in adults in Senegal. The leaves and the bark of the root are used as anti-dysentery while the stem bark for the treatment of tuberculin cough and the yellow pulp of root and the black leaves are used as dye. The scented heart wood is used as perfume called "amu" and the root bark is used to treat wound and strains. The macerated stem bark serves as antiseptic to wash mouth in order to resist gingivitis, thrush and general body pains in Congo. The plant also serves as wound dressing, diuretic management, pile and yaws treatment (Ivory coast), anti-skin inflammation, sores and ulcers treatment (Sierra Leone), eye lotion (Gambia), hair perfume, severe jaundice and chewing stick (Cameroon) across other African countries (Savil and Fox, 1967; Kerharo and Adam, 1974; Abbow, 1990). It's

also known that this plant synthesizes derivatives useful for the maintenance of health in human and animals (Srivastaraj and Vietineyer, 1996; Tapsell, 2006). Records have shown that a few reports are available on the antimicrobial potentials of this African plant of rich heritage. Root bark of *T. laxiflora* displayed bactericidal activity by both aqueous and ethanolic extracts for several human pathogens. Extracts from *T. laxiflora*, if developed into drug, could be safely administered across ages (i.e young, old, infant, male, female, pregnant or aged). Local use of the stem as chewing stick will help to dislodge and disinfect the microbes in the gingival creeks and gum of the tooth and prevent weakness of the gum. Also, the development and production of antibiotics from this plant as herbal drug for all and sundry will be significant efforts towards controlling diarrhoea among the developing nations (Fasola et al., 2013).

In Sudan decoction of *T. laxiflora* stem bark are used for malaria and cough treatments (Musa et al., 2011). Women also use heartwood for fumigant "smoke bath". Exposure to the smoke bath is believed to relieve rheumatic pain, smooth skin and achieve general body relaxation beside other cosmetic and medicinal beautification (Ogbazghi and Bein, 2006). Earlier work done on the root bark of *T. laxiflora* has led to isolation of several compounds like laxiflorin, ellagic acid, trimethylellagic acid, tetramethylellagic acid and terminolic acid (Ekong and Idemudia, 1967). Methanol 80% extract of *T. laxiflora* leaves led to isolation of beta-sitosterol, three hydrolysable tannins (methyl gallate, gallic acid and ellagic acid) and five flavonoids; quercetin, vitexin, isovitexin, quercetin 3-O-α-rhamnoside and rutin (Rashed and Ono, 2013)

In this study four compounds from methanolic wood extract of *T. laxiflora* have been isolated and evaluated for their ani-acne activities using antibacterial assay

against *P. acnes*, a lipase inhibitory assay and l,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay

3.2 Materials and methods

3.2.1 Plant materials

T. laxiflora wood was purchased from Khartoum, Sudan in March 2011. The specimen was authenticated by Dr. Ashraf Mohamed (Faculty of Forest, University of Khartoum). Voucher specimen (SD-KH-03) was deposited at the Department of Horticulture, Faculty of Agriculture, University of Khartoum.

3.2.2 Extraction and isolation

T. laxiflora wood (300 g) was air-dried at room temperature, powdered and extracted with 3L of methanol for 12 h three times. The extract was filtered then the solvent was removed under vacuum using rotary evaporator at 30ºC. The yield of methanol extract was 42.9 g (14.3%).

Part of the extract (10 g) was separated by medium pressure liquid chromatography (MPLC) using ODS column (YMC-DispoPack AT ODS-25:120 g).The column was conditioned with the first eluent used for separation for 30 min. with flow rate 0.5 ml/min to ensure that no other contaminations were present on the column. MPLC separation was performed by using a chromatography pump (540 Yamazen, Japan), UV detector at 280 nm wavelength (UV-10V Yamazen, Japan) and fraction collector (SF-2120, Advantec Tokyo Ltd, Japan).

Elution with H_2O/MeOH =95/5 (v/v) and 20/80 (v/v) gave two fractions (**F_1 and F_2)**. Fraction one (**F_1**) (200 mg) was subjected to column chromatography on a Sephadex LH-20 using MeOH and 70% acetone in water (v/v) as the eluent to give four

fractions (F1-1 to F1-4). Separation of these sub-fractions (F1-1, F1-2 and F1-4) were performed using preparative high performance liquid chromatography (HPLC) with reversed phase Inertsil OD-3 column (GL Sciences Inc. 10 mm i.d.× 250 mm) monitored at 280 nm. The solvent system used was as follows: a gradient program for 60 min from 10–100% methanol in water with 0.05% TFA at a flow rate 5 ml/min. This separation gave gallic acid (10 mg), flavogallonic acid dilactone (27.5 mg) and terchublin (16.9 mg). Fraction two (**F_2**) was also subjected to preparative HPLC under the same condition to isolate ellagic acid (35 mg).

3.2.3 Identification of isolated compounds from *T. laxiflora*

Compounds were identified by ^{1}H ,^{13}C nuclear magnetic resonance (NMR) and Liquid chromatography – mass spectrometry (LC-MS). Methanol-d_4 was used as the NMR solvent. NMR measurements were obtained by using JEOL ECP 600 MHz NMR. LC-MS (Waters Waters®XevoTM QTof MS) measurements were performed using column C_{18} (2.1mm i.d. × 100 mm) with MeOH/H_2O = 5/95 (v/v) (30 min), 100/0 (10 min) with a linear gradient as elunt. The data were collected in negative ionization mode. Spectroscopic data of flavogallonic acid dilactone (Fig 3.3) and terchublin (Fig 3.4) are known from the literature. Gallic acid (Fig 3.1) and ellagic acid (Fig 3.2) were identified by comparing the spectroscopic data of commercial reagents.

3.2.4 Bioassay methods

Antimicrobial activity

Antimicrobial activity was performed as described in Chapter 2.

Antioxidant activity

Antioxidant activity was performed as described in Chapter 2.

Lipase inhibitory activity

Lipase inhibitory activity was performed as described in Chapter 2.

3.3 Results and discussion

Bioassay-guided fractionation is a procedure of whereby extract is chromatographically fractionated and re-fractionated until a pure biologically active compound is isolated. Each fraction produced during the fractionation process is evaluated in a bioassay system and only active fractions are fractionated. Bioassay-guided fractionation method is commonly employed in drug discovery research due to its effectiveness to directly linked the analyzed extract and targeted compounds using fractionation procedure that followed with certain biological activity.

3.3.1 The antimicrobial activity

P. acnes, an anaerobic pathogenic inhabitant of human skin, plays an important role in pathogenesis of acne. For many years antibiotics and hormones were usually applied to treat acne (Poulin, 2004; Tan and Tan, 2005). However, these agents are often accompanied by severe side effects and drug resistance (Leyden, 2004; Yemisci et al., 2005). Therefore, many researchers have tried to develop therapeutic agents for acne that have less side effects and high antibacterial activity. Antibacterial activities of the compounds isolated from *T. laxiflora* towards *P. acnes* were analyzed. In Table (3.1) MIC and MBC data indicated that terchebulin exhibited the strongest inhibitory activities (MIC=125 µg/ml and MBC=250 µg/ml). Silva et al., (2012) reported that the ellagitannins terchebulin and punicalagin isolated from *T. macroptera* root had anti-*Helicobacter pylori* activity with MIC value 200 µg/ml. In addition chebulagic acid isolated from the fruit of *T. chebula* showed inhibitory activity against *P. acnes* with MIC value of 12.5 µg/ml (Patil et al., 2012). On the other hand ellagic acid showed

good MIC value (125 μg/ml), with low MBC value (1000 μg/ml). According to Panichayupakaranant (2010) ellagic acid-rich pomegranate rind extracts exhibited potent bacteriostatic effect against *P. acnes* with an MIC value of 15.6μg/ml. The antibacterial activities of these four compounds were lower than tetracycline. However the effectiveness of terchebulin, ellagic acid and flavogallonic acid dilactone are better than Isopropyl methylphenol (IPMP) as positive control. Gallic acid showed low MIC and MBC value of 2000 μg/ml. These values are consistent with the result previously reported (Patil et al., 2012).

3.3.2 Lipase inhibitory activity

Earlier studies have suggested that the fatty acids produced by lipase activity from *p. acnes* resulted in inflammation owing to neutrophil chemotaxis (Lee et al., 1982). A decline in free fatty acids caused by lipase inhibition is associated with a decrease in the growth of *P. acnes* (Strauss and Pochi, 1965). Natural inhibitors of lipase such as flavonoids were reported as promising candidates for acne treatment (Falcocchio et al., 2006). In addition therapeutic agents for acne like antibiotics are usually employed to inhibit inflammation or kill the bacteria. Several reports also suggest that in case of tetracycline, erythromycin and clindamycin several side effects were observed such as appearance of resistant bacteria, organ damage and immunohypersensitivity if they have been taken for a long time (Wawruch et al., 2002). In the present study lipase inhibitory activity of isolated compounds were also evaluated. The results in Table (3.1) revealed that gallic acid and terchebulin have anti-lipase activity with IC_{50} value of 149.3 and 260.7 μM respectively. Previously inhibitory activities of Kaempferol and glycyrrhizic acid on *P. acnes* lipase has been reported and there IC_{50} (240-340 μM) were evaluated fairly high lipase inhibitors (Falcocchio et al.,

2006). In comparison with the above, this study has indicated that inhibitory level of terchebulin is less than gallic acid however it's still in the range of natural inhibitor of lipase. These results are in contradiction to that of Patil et al. (2012) who stated that gallic acid did not inhibit lipase activity of *p. acnes* (with IC_{50} > 5000 μM), furthermore he mentioned that *T. chebula* fruits extract showed significant inhibition of lipase activity (IC_{50} =60 μM). Various studies support our results that gallic acid possess anti-lipase activity but specifically for pancreatic lipase (Oi et al., 2012). Makihara et al. (2012) concluded that gallic acid is the active ingredient of *T. bellirica* fruits that causes pancreatic lipase inhibition. Although several enzymatic activities are affected by ellagitannin-related compounds and could be influenced by the number and orientation of the galloyl group (Hirano et al., 2003), it was a surprise for us to report that gallic acid alone could have high activity. This inhibition of lipase by this compound, might be due to the binding of the substrate or the interaction with enzyme. Further research is needed to determine the mechanism of inhibition induced by these compounds. The IC_{50} values of flavogallonic acid dilactone and ellagic acid were (534.80 and 832.59 μM respectively). Although tetracycline as potent antimicrobial agent, it has inhibitory activity against *p. acnes* lipase and it's used as positive control (Batubara et al. 2010; Patil et al., 2012). Tetracycline has low lipase inhibitory activity (883.3 μM) as compared to gallic acid, terchebulin and flavogallonic acid dilactone. Previously Batubara et al. also reported that brazilin isolated from *Caesalpinia sappan* L. wood had strong lipase inhibition than tetracycline (Batubara et al., 2010). To our knowledge this is the first time to investigate the anti-lipase activity of terchebulin and flavogallonic acid dilactone.

3.3.3 The antioxidant activity

Reactive oxygen species (ROS) including singlet oxygen, superoxide anion, hydrogen peroxide, lipid peroxide and nitric oxide play an important role in inflammatory acnes as well as tissue injury. ROS promote tumor necrosis factor formation (Trefzer et al., 1993) and consequently activate T lymphocytes and keratinocytes. Also the cytokines and other proinflammatory compounds are produced and released causing microcomedones. These microcomedones further develop in to comedones and inflammatory lesions (Kanlayavattanakul and Lourith, 2011). ROS are subsequently generated from the hyper-colonization of *P. acnes* and from ultraviolet exposure (Leyden, 2001). Chemotactic substances released from the bacteria attract polymorphonuclear leukocytes to the site of inflammation (Vowels et al., 1995). After phagocytosis of the bacteria, the attracted neutrophils are thought to release lysosomal enzymes and produce (ROS) that can damage the follicular epithelium. Beside that stimulating of epidermal cells by *P. acnes* leading to generate ROS particularly superoxide anion. This phenomenon is associated with production of a soluble proinflammatory molecule and epidermal cell death (Grange et al., 2009)

Therefore compounds that inhibit the growth of skin microorganisms and have antioxidant activity are required. We examined the isolated compounds for their antioxidant activities using DPPH radical scavenging assay as shown in Table (3.1), ellagic acid and terchebulin exhibited strong antioxidant activity with IC_{50} 4.86 μM and 4.90 μM respectively, when compared to positive control of (+)-catechin (IC_{50} 8.23 μM). However flavogallonic acid dilactone and gallic acid showed relatively similar activity to (+)-catechin (IC_{50} 7.03 μM and 8.90 μM respectively). Yokozawa et al. (1998) reported that most of the tannins with low concentration have high activity than flavonoids on DPPH radical scavenging. An increase in galloyl group, molecular weight

and ortho-hydroxyl structure enhanced the antioxidant activity of tannins. Antioxidant activity using DPPH radical scavenging, oxygen radical absorbance capacity (ORAC) and ferric reducing ability of plasma (FRAP) in vitro assays indicated that chebulic ellagitannins have high antioxidant activity within isolated compounds from three *Terminalia sp*. (Pfundstein et al., 2010). Manosroie et al., (2010) showed that isoterchebulin and other hydrolyzable tannins isolated from galls of *T. chebula* had strong radical scavenging activity.

3.4 Conclusion

From the data presented in this study, it is evident that some isolated compounds such as ellagic acid, gallic acid and flavogallonic acid dilactone from the methanolic extracts of *T. laxiflora* wood have potentiality as anti-acne agent. Although terchebulin showed excellent potency as anti-acne agent, since it demonstrated antibacterial activity against *P. acnes* (MIC=125 µg/ml MBC= 250 µg/ml) with strong antioxidant activity and good lipase inhibitory activity.

Table 3.1. Anti-*Propionibacterium acnes*, Lipase inhibitory and antioxidant activities of isolated compounds from *T. laxiflora*

Sample	MIC[a] (μg/ml)	MBC[b] (μg/ml)	Lipase inhibition IC_{50}[c] μM	Antioxidant IC_{50}(μM)
Gallic acid	2000	2000	149.31±10.9	8.90±0.30
Ellagic acid	125	1000	832.59±7.09	4.86±0.03
Flavogallonic acid dilactone	250	1000	534.80±6.99	7.03±0.43
Terchebulin	125	250	260.65±1.60	4.90±0.10
Tetracycline	0.03	0.122	883.30±4.94	nd
IPMP[d]	1000	2000	＞3328.45	nd
(+)-Catechin	nd[e]	nd	＞1722.53	8.23±0.12

[a] MIC: minimal inhibitory concentration

[b] MBC: minimal bactericidal concentration

[c] IC_{50}: 50% inhibitory concentration

[d] IPMP: 3-methyl 4-isopropylphenol

[e] nd: not determined

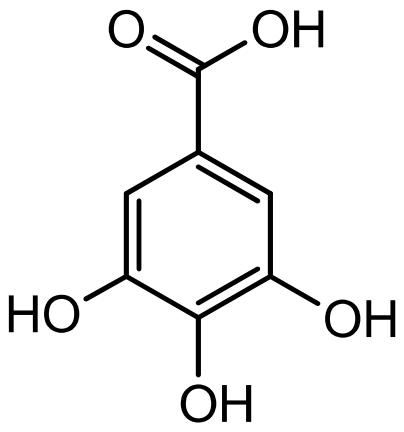

Fig 3.1. Gallic acid

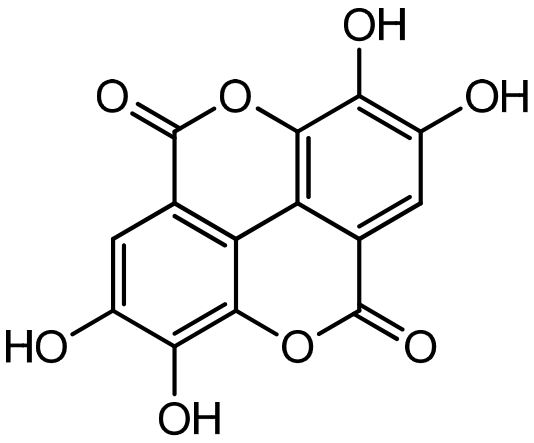

Fig 3.2. Ellagic acid

Flavogallonic acid dilactone: A tan powder. LC-MS (negative ion mode) *m/z*:469 (M-H); ^{1}H-NMR (in CD_3OD): δ (ppm) 7.26 (s), 7.50 (s). ^{13}C-NMR (in CD_3OD): δ (ppm) 108.1 (C-1, 1′), 110.1-114.4 (C-6, 6′), 112.8 (C-5), 113.3 (C-6″) , 117.5-120.2 (C-5′, 2″), 124.9 (C-1″) , 135.7 (C-2), 136.3 (C-3), 136.5 (C-4) , 137.8 (C-2′), 139.2 (C-3′), 143.2 (C-4′), 144.1 (C-3″), 145.9 (C-4″), 147.8 (C-5″), 158.9-160.4 (C-7, 7′), 168.9(C-7″).

^{1}H-NMR and ^{13}C-NMR spectral data were coincided to that of published report (Hirano et al., 2003)

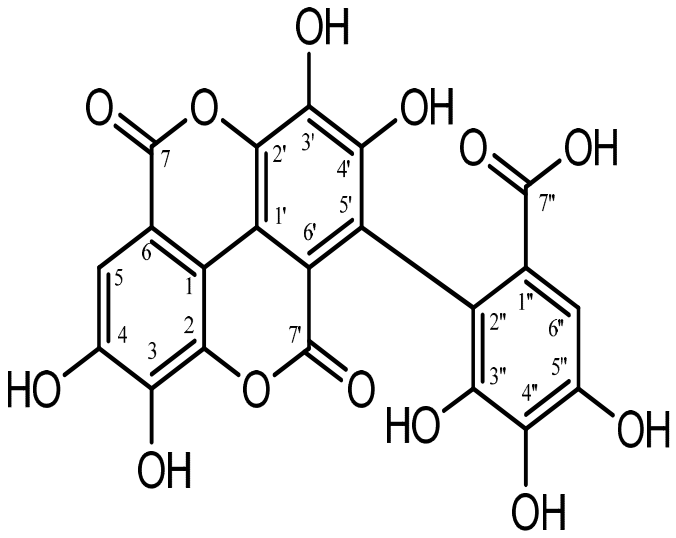

Fig 3.3. Flavogallonic acid dilactone

Terchebulin: Tan powder. LC-MS (negative ion mode) *m/z*: 1083 (M-H); ^{1}H-NMR (in CD_3OD): δ (ppm) 3.04 (t, *J*=11.6 Hz, one of the H-6″), 4.21 (t, *J*=10.3 Hz, H-5″), 4.48 (t, *J*=8.9 Hz, one of the H-6″), 4.78 (t, *J*=11.0 Hz, H-4″), 4.98 (dd, *J*= 3.5, 9.7 Hz, H-2″), 5.23 (d, *J*=2.8 Hz, H-1″), 5.64 (t, *J*= 9.6 Hz, H-3″), 6.37 (s, H-B6), 6.42 (s, H-D6), 6.56 (s, H-A2), 6.79 (s, H-C2), 7.48 (s, H-5). ^{13}C-NMR (in CD_3OD): δ (ppm) 63.4 (C-6″), 68.5 (C-4″), 69.0 (C-5″), 74.1 (C-3″), 74.2 (C-2″), 90.2 (C-1″), 106.4 (C-B6), 106.5 (C-D6), 106.8 (C-A2), 108.5(C-C2), 112.0-114.0 (C-A6, B2, 5, 5′, 1, 1′, 2, 2′, 6, 6′), 116.0 (C-C6), 122.2 (C-D1), 123.5 (C- B1,C1), 125.1 (C-A1), 135.9 (C-B4), 136.1 (C-A4), 137.5 (C-C4), 137.6 (C-D4), 138.4 (C-3), 139.1 (C-D3), 140.7 (C-3′), 141.7 (C-D2), 143.4-143.6 (C-A5, B3, C5), 144.5-144.6 (C-A3, B5, C3, D5), 147.4 (C-4′), 150.3 (C-4), 158.3 (C-7′), 159.5 (C-7), 166.9 (C-D7), 167.0 (C-C7), 168.9 (C-A7), 169.5 (C-B7).

^{1}H-NMR and ^{13}C-NMR spectral data were coincided to that of published report (Lin et al., 1990)

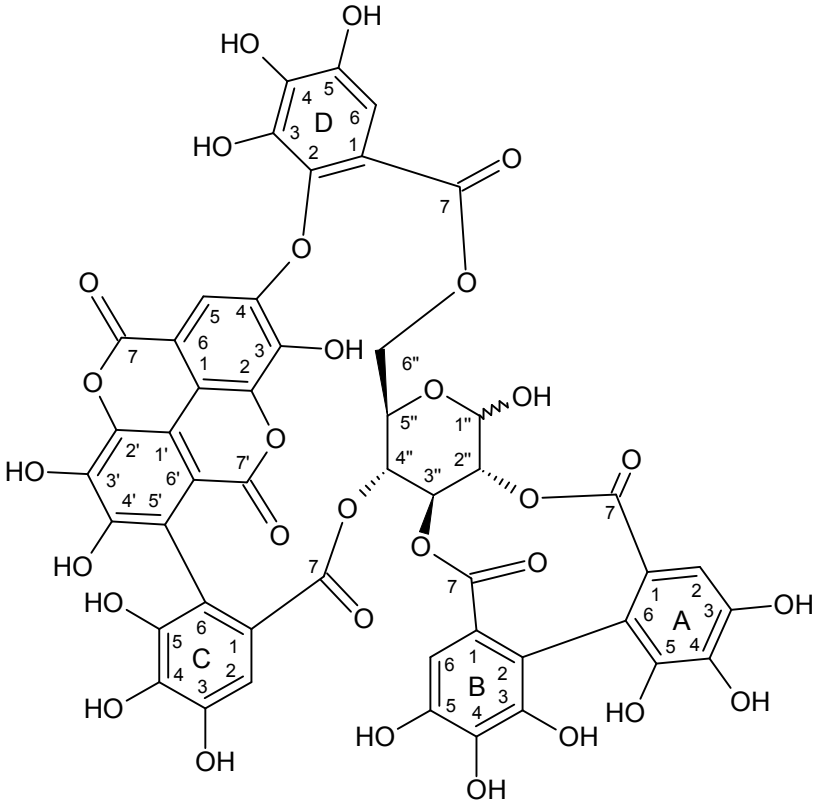

Fig 3.4. Terchebulin

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Chapter 4
Potency of *Acacia nilotica* (L.) Willd. ex Delile subsp. nilotica pods as anti-acne agent
General conclusion and perspectives

4.1 Introduction

Acne is one of the most common dermatological disorders, seen mainly in adolescents but affecting the adult population to some extent, as well. The prevalence of acne is reported to range from 30-85% in adolescents and young adults (Dreno and Poli, 2003). Various psychiatric conditions like depression, low self-esteem and social anxiety have previously been postulated to be common among these patients (Cotterill and Cunliffe, 1997; Smithard et al., 2001). The pathogenesis of acne is complex but dependent on four key factors including androgen-mediated stimulation of sebaceous gland activity, follicular hyperkeratinization, colonization of the bacterium *P. acnes* (an anaerobic bacterium as a normal constituent of the skin microbial flora), and inflammation (Toyoda and Morohashi, 2001). The high levels of sebum elicited by androgen cause proliferation of *P. acnes* in the pilosebaceous ducts and this proliferation triggers the host inflammatory response with a discharge of the proinflammatory cytokines, interleukin-1b (IL-1b), IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF) and TNF-α (Gollnick et al., 2003)

Recent studies on the aetiopathogenesis of acne vulgaris have focused on the role of oxygen free radicals and intracellular antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), (Arican et al., 2005; Kurutas et al., 2005). If antioxidant enzymes become incapable in oxidative damage, oxygen free radicals initiate lipid peroxidation in cell and organelle membranes. It has been reported that oxygen free radicals, which are generated by the neutrophils on the follicular wall to kill microorganisms, may cause cell damage at the site of inflammation (Zouboulis et al., 2005; Akamatsu et al., 2003). Sarici et al., (2010) stated that decrease in antioxidants enzyme such as SOD, and CAT in acne patients, it can be concluded that oxidative

stress plays a role in the pathogenesis of acne vulgaris, therefore antioxidant drugs can be indicated for treatment of acne. The success of some drugs like metronidazole in acne treatment could also be at least partially attributed to its inhibition of ROS generation. On the other hand, benzoyl peroxide a topical agent for the acne treatment, shows antibacterial activity, in addition it has ability to induce an inflammatory reaction mediated by oxidative stress in HaCaT keratinocytes cell (Valacchi et al., 2001).

Lipases play an important role in pathogenesis of acne by hydrolyzing sebum triglycerides and releasing irritating free fatty acids in the pilosebaceous follicles, which is one of the major causes of lesions in acne vulgaris (Marples et al., 1971). Furthermore, antibiotics such as tetracycline, erythromycin and propylene phenoxetol have been shown to inhibit *P. acnes* lipase activity (Uncles and Gemmell, 1982; Webster, 1981). A decline in free fatty acids caused by lipase inhibition is associated with a decrease in the growth of *P. acn*es (Strauss and Pochi, 1965). Therefore, lipase has generated a high interest as a pharmacological target for anti-acne drugs.

4.1.1 The genus of Acacia sp.

Acacia Mill. is a widespread genus of tropical-subtropical trees and shrubs ranging from central/ south America through Africa to south-east Asia and Australia (Ross, 1981). The genus of Acacia species currently include about 1300 species and form the second-most species-rich genus in the family Leguminosae (Mabberley, 1997; Maslin, 2001). Two third of its species are indigenous to Australia which makes Acacia the largest genus of vascular plants in the region (Maslin, 2001). Acacia species (31 species) predominate in Sudan and are of high importance not only because of the gum produced by some of them but also for their medicinal and economical importance. They represent about one third of the African species (Elamin, 1972).

4.1.2 *Acacia nilotica* (L.) Willd. ex Delile subsp. nilotica

(a)  (b) 

Fig 4.1. *Acacia nilotica* (a) pods and (b) seeds

(Attenborough, 1995)

Acacia nilotica subsp. nilotica (sunt in Arabic) is a tree 5-20 m high with a dense spherical crown, stems and branches usually dark to black colored, fissured bark, grey-pinkish slash, exuding a reddish low quality gum. The tree has thin, straight, light, grey spines in axillaries pairs, usually in 3 to 12 pairs, 5 to 7.5 cm long in young trees, mature trees commonly without thorns (Fig 4.3). The leaves are bipinnate, with 3-6 pairs of pinnulae and 10-30 pairs of leaflets each, tomentose, rachis with a gland at the bottom of the last pair of pinnulae. Flowers in globulous heads 1.2-1.5 cm in diameter of a bright golden-yellow color set up either axillaries or whorl on peduncles 2-3 cm long located at the end of the branches. The tree found in the central and northern parts of the Sudan and Egypt and is known in Sudanese folk medicine by the common name "Kaarad"

Pods are strongly constricted, hairy, white-grey, thick and softly tomentose. Its seeds number approximately 8000/kg (Fagg, 2001). Seeds are extremely hard-coated. They are oblong, 6.5-11 mm long, 12-14 mm wide and 3.5-4 cm thick, dark brown to blackish brown (Fig 4.1-b). Pleurogram distinct oblong creamy white (Fig 4.1-a). *A. nilotica* belongs to family Fabaceae, subfamily Mimosoideae, tribe Acacieae, genus Acacia, species *A. nilotica*, and subspecies nilotica. Nine subspecies/varieties are

recognized, the most important are subsp. adstringens; subsp. kraussiana; subsp. nilotica; and subsp. tomentosa (Kongevej, 2008).

4.1.3 The non medicinal use of *A. nilotica*

A. nilotica is one of the most important multipurpose tree species of dry land in Africa and South Asia. The wood is hard and used for general construction purposes and implements. It is fairly termite resistant. The wood yields an excellent fuel wood. *A. nilotica* wood, as species considered to be one of the most important tree species under management in the Sudan. It is the most dominant hardwood species on the banks of the Blue Nile and White Nile (El Amin and Hamza, 1990). The dominance of this species allowed establishing sawmills plants at El Suki and Wad en Naial area. The plants produce mainly railway sleepers, other sawn products and firewood

Flowers are attracting bees and make a good base for apiculture. Pods are nutritious and make a high quality fodder for livestock especially during the dry season. The pods and leaves are very popular with cattle. Pods are also used as a supplement to poultry rations in India (Fagg, 2001). *A. nilotica* makes a good protective hedge because of its thorns. In Ethiopia certain parts of the tree are used as galactagogue.

4.1.4 The medicinal use and chemical constituents of *A. nilotica*

Most of the Acacias are of medicinal and health benefits to human being. For example, *A. nilotica* are used in treatment of wound (pods), malaria, sore throat and (aerial part and pod) and toothache (bark) (Jain et al., 2005; Kubmarawa et al., 2007) while Gum Arabic is applied for kidney diseases treatment.

Also, *A. nilotica* be used as medicine for African Zulu take the (bark) for cough. Furthermore, it acts as an astringent and it is used to treat diarrhea, dysentery, and

leprosy. In West Africa, the bark or gum is used to treat cancers and/or tumors (of ear, eye, or testicles) and indurations of liver and spleen and condylomas. Sap or bark, leaves, and young pods are strongly astringent due to tannin, and are chewed in Senegal as an antiscorbutic. The bruised leaves are poulticed and used to treat ulcers. In Lebanon the resin is mixed with orange flower infusion to typhoid convalescence. In Tonga, The root is used to treat tuberculosis. Egyptian Nubians believe that diabetics may eat unlimited carbohydrates as long as they also consume powdered pods (Fagg, 2001).

Tannin as group of complex hydrocarbon substance in the plants, consist of two types of polyphenolic systems, namely hydrolysable or condensed. Most of the Acacias produce tannins. *A. nilotica* for instance, produces tannins, especially the inner bark, which is used commercially for tanning and dyeing leather black in Sudan (Sahni, 1968). Fruits and/or bark of the three Sudanese subspecies of *A. nilotica* contain more than 10% tannin. The tannins of the *A. nilotica* subspecies are of the hydrolysable-condensed types while that of *A. mearnsii* (wattle) is the condensed type (Ahmed et al. 2005). *Acacia* species is considered as a rich source of gallic and ellagic acid (Sultana et al., 2010). Many researchers have indicated that tannins and related compounds have different astringent effect (Varro, 1994) and inhibitory effect on pathogens (Hubbes, 1962). This accounts for their medicinal properties. Accordingly, pods of *A. nilotica* were found to have, molluscicidal (Ayoub, 1985), algicidal (Ayoub, 1984), anti-protozoa, antibacterial (Massele and Nshimol, 1995) and antifungal effects (Nath D et al., 1995).

The pod is also reported to exhibit anti-inflammatory (Dafallah and Al-Mustafa, 1996) and antiplatelet activities (Shah et al., 1997). Methanol (bark and pods) and aqueous (pods) extracts are reported to exert inhibitory effects against HIV-1 protease

(Hussein et al., 1999) and hepatitis C virus protease (Hussein et al., 2000). Phytochemical studies of the aerial parts of the plant resulted in the identification of a variety of phenolic constituents, among which catechin derivatives were identified (El-Sayyad, 1979; Singh, 2008). These compounds have a wide range of biological activities, in particular antioxidant, anticarcinogenic and anti-inflammatory activities (Santos-Buelga and Scalbert, 2005). Anti-inflammatory activity described for these molecules is probably related to their antioxidant properties (Goncalves et al., 2005). Maldini et al., (2011) purifed galloylated catechin- and gallocatechin derivatives along with galloylated glucose derivatives from 80% EtOH extract of *A. nilotica* pods. The isolated compounds included; 1,3-di-O-galloyl- β -D- glucopyranose (1), di-O-galloyl- β -D- glucopyranose (2), gallic acid methyl ester (3), galloctechin -7-O-gallate (4), gallic acid methylester-4-gallate (5), gallocatechin-7,3'-di-O-gallate (6), gallocatechin-7,4'-di-O-gallate (7), catechin-7-O-gallate (8), catechin-7,3'-di-O-gallate (9) and catechin-7,4'-di-O-gallate (10) (Fig 4.2).

Based on previous screen data, we found that the methanol extracts of *A. nilotica* (L.) pods showed the best DPPH radical scavenging activity, lipase inhibitory activity and weak antimicrobial activity. The aim of this chapter to identify the active compounds from *A. nilotica* extracts conferring it good antioxidant activity and anti- *p. acne* lipase. Thus we performed successive extraction and bioassay guided fractionation for the best extract.

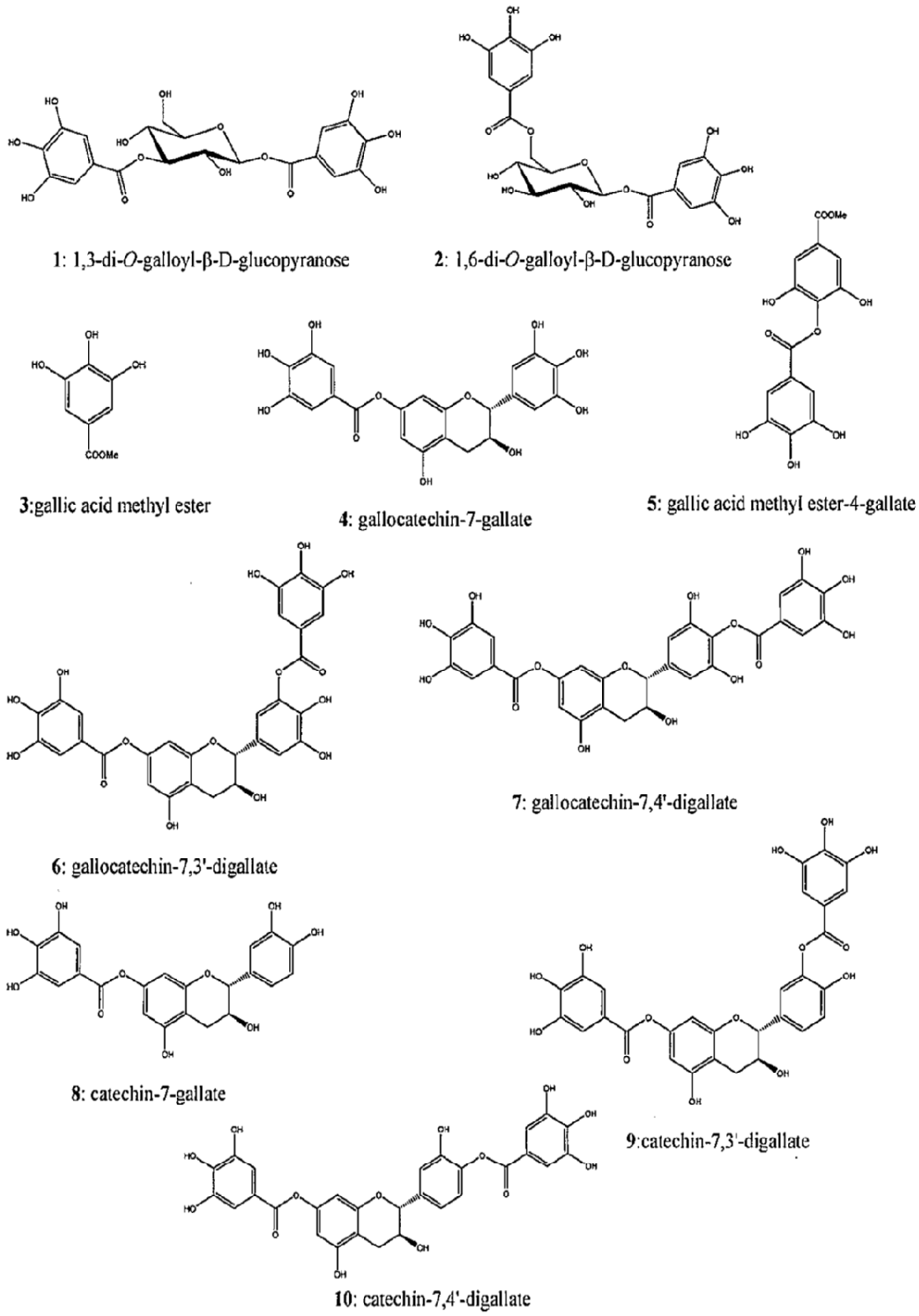


Fig 4.2. Compounds 1–10 from *A. nilotica* pods

Source: Maldini et al., (2011)

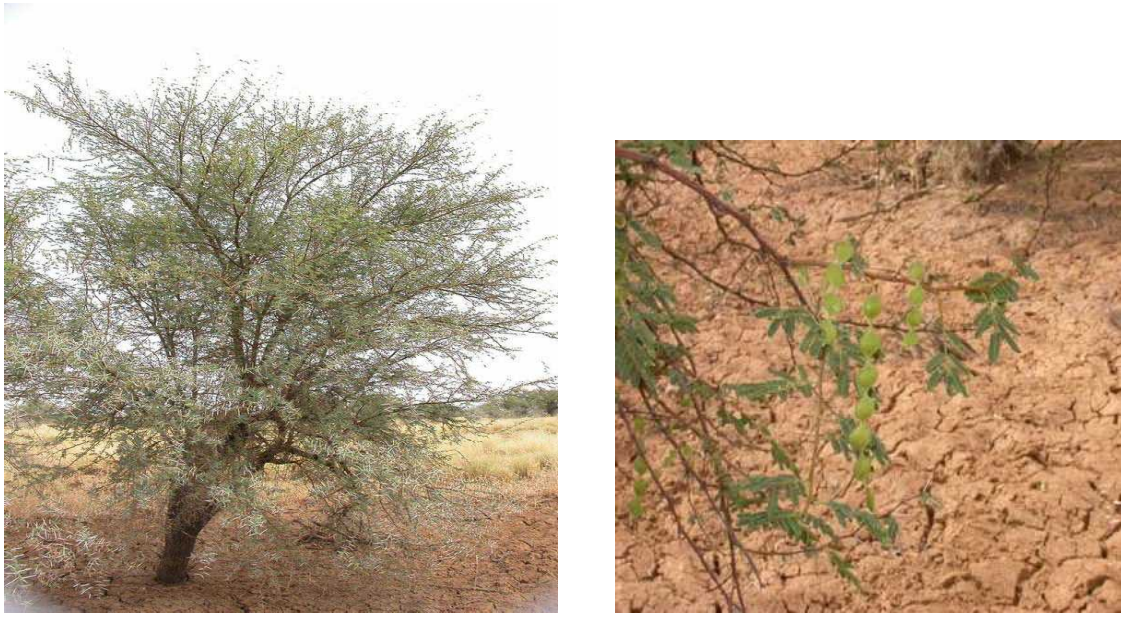


Fig 4.3. *Acacia nilotica*

4.2 Material and Methods

4.2.1 Plant materials

A. nilotica (L.) pods was purchased from Khartoum (Omdurman market), Sudan on March 2011. Voucher specimens (SD-OD-01) were deposited in the Horticultural Laboratory, Department of Horticulture, Faculty of Agriculture, University of Khartoum.

4.2.2 Preparation of plant extracts

A. nilotica (L.) pods (30 g) was dried and ground before using maceration for 24 h for successive extraction (depending on the increasing of polarity), which was performed based on scheme as in Fig (4.4). The dried and powdered pods were extracted with solvents (ratio 1 g sample: 10 ml solvent) for three times. The extracts were filtered using Whatman filter paper (no.1) and concentrated in vacuo at 30ºC using a rotary evaporator. Then each solvent extracts were subjected to HPLC analysis (Fig 4.9).

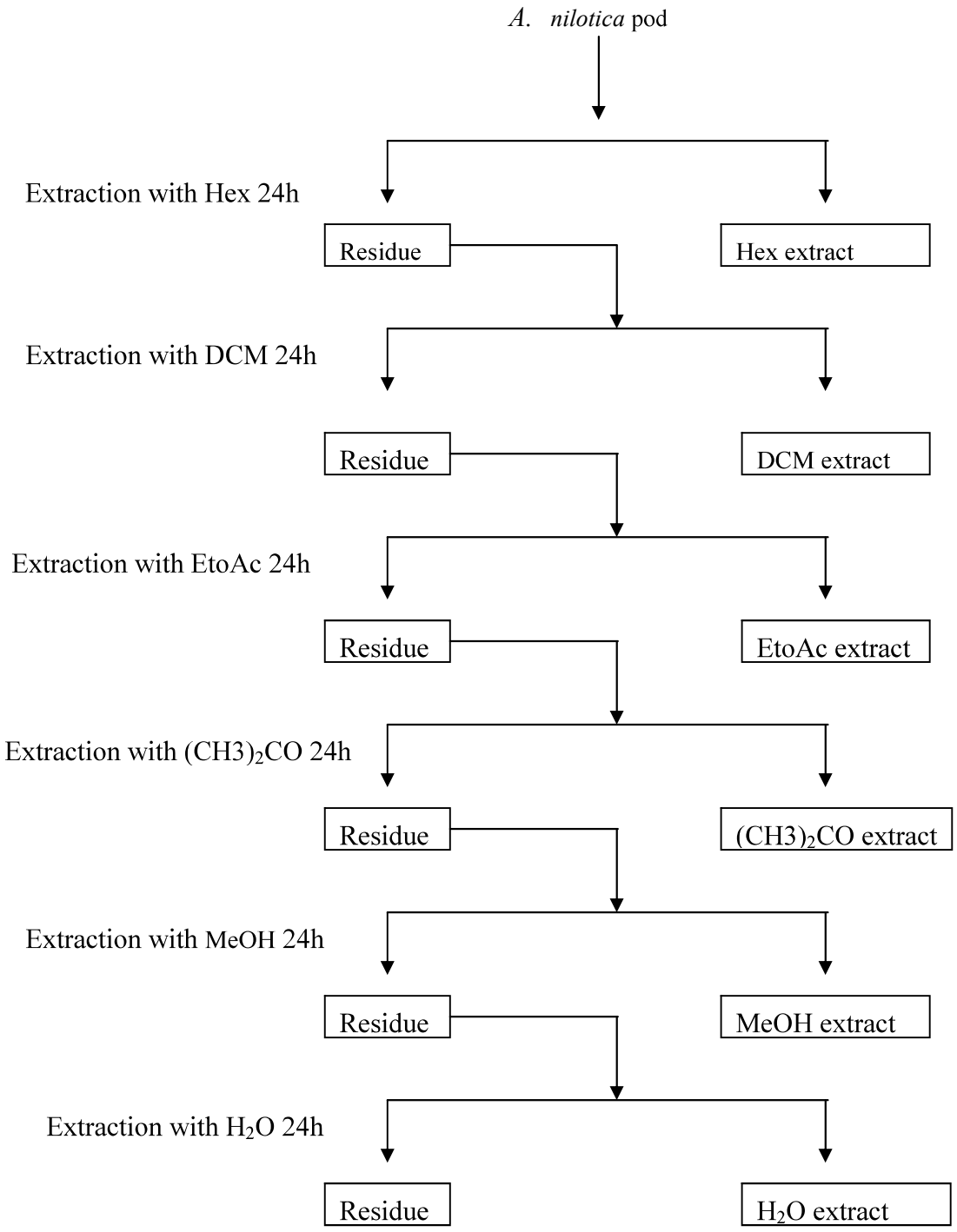
4.2.3 HPLC analysis

HPLC analysis was performed to all extracts that was produced. The condition of HPLC are using; column: Sunniest C_{18}, 5µm (4.5Ø X 250 mm). 1.0 ml/ min flow rate, analysis time: 65 min., column temperature 40ºC, UV detector at 280nm. Gradient program: MeoH: 0.05% TFA water= 5%:95% (10 min.), 100%:0% (55 min.).

4.2.4 Fractionation of *A. nilotica* pod of methanolic extracts

Part of methanol extract (1 g) was separated by medium pressure liquid chromatography (MPLC). ODS column (YMC-DispoPack AT ODS-25:120 g), and an automatic fraction collector was used. The column was preconditioned with water/ methanol for 30 min. with flow rate 4 ml/min to ensure that no other contamination was present on the column. The column was then eluted with series of solvent system (eluent 1) water/methanol (95:5 v/v), (eluent 2) water/methanol (80:20 v/v) and (eluent 3) water/methanol (50:50 v/v) to determine the best elution system for separation the compounds (Fig 4.5).

In elute (1) one fraction was collected. Then in elute (3) eight fractions were collected. All this fractions were subjected to HPLC analysis (Fig 4.10). Standard solution of expected compound was prepared (methyl gallate, Wako pure chemical com. Ltd) & (gallic acid, Naka Lai Tesque, INC) and analysis by HPLC. The peaks were detected at 280 nm. The retention time of methyl gallate and gallic acid was 17.8 and 9.8 min. respectively.



Hex: Hexane ; DCM: dichloromethane ; EtoAc: Ethylacetate ;$(CH3)_2CO$: Acetone ; MeOH :Methanol; H_2O: Water

Fig 4.4. Extraction of *A. nilotica* pod by different solvents

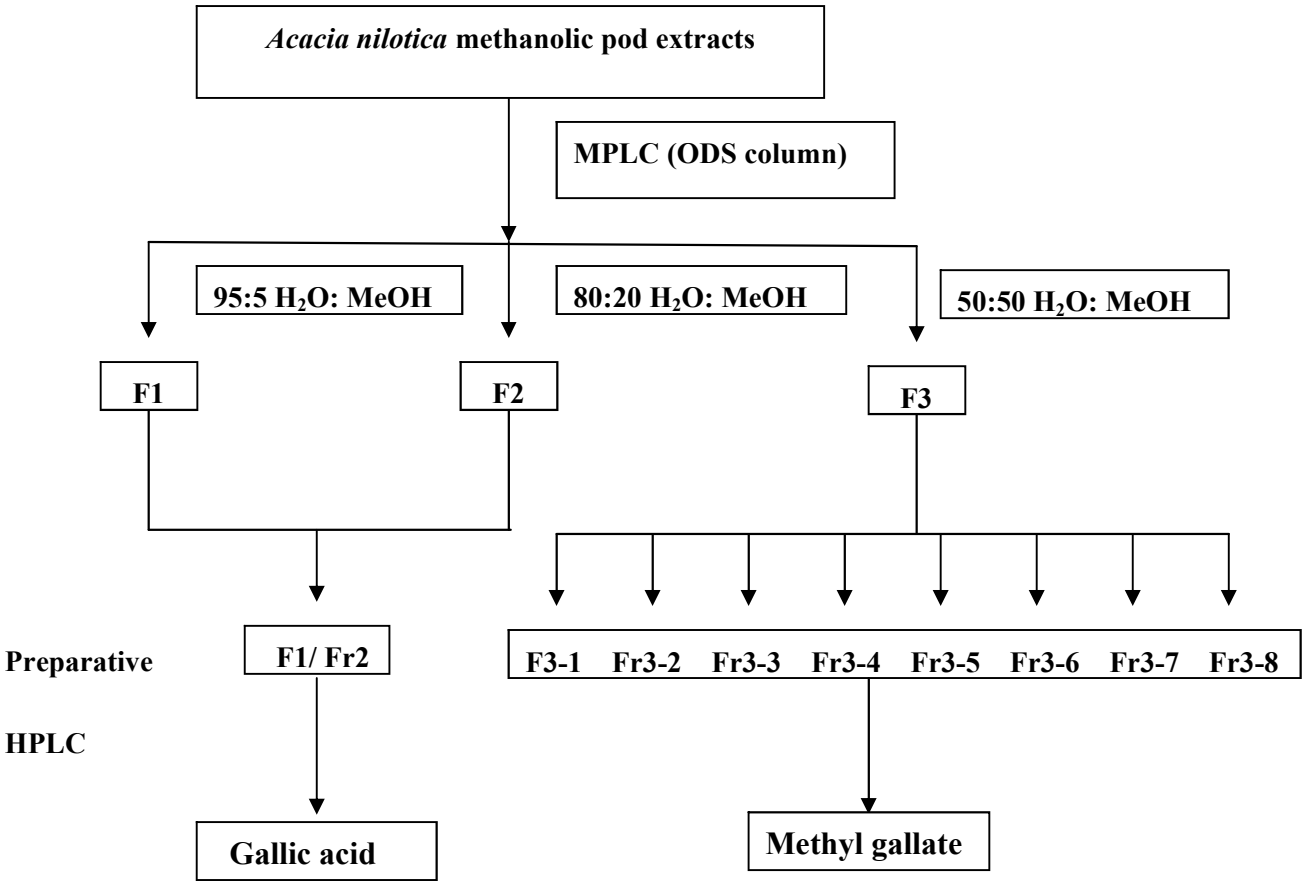


Fig 4.5. Scheme for fractionation of *A. nilotica* pod methanolic extract

4.2.5 Bioassay methods

Antimicrobial activity

Antimicrobial activity was performed as described in Chapter 2.

Antioxidant activity

Antioxidant activity was performed as described in Chapter 2.

Lipase inhibitory activity

Lipase inhibitory activity was performed as described in Chapter 2.

4.3 Result and discussion

The antibacterial activities of all extracts against *P. acnes* and DPPH radical-scavenging activity are presented in Table (4.1). The result showed that Hexane, dichloromethane and water extracts had no activity at the concentration of 4 mg/ml. Methanol showed anti-*P. acnes* activity with MIC value of 4 mg/ml. Acetone and Ethyl acetate extracts offered anti-*P. acnes* activity with MIC value of 2 mg/ml. In earlier studies, extracts of the *A. nilotica* pods displayed antibacterial activity against *Staphylococcus aureus* with MIC value of 0.4 mg/ml. Abdel Nabi et al., (1992) suggested that the antimicrobial activity observed is not due to tannins but to another substance(s). Methyl gallate and gallic acid, showed anti-*p. acnes* activity with MIC value of 2 mg/ml. Methyl gallate and gallic acid isolated from pomegranate peel showed antibacterial activity against ten clinical isolates of *Staphylococcus aureus* (methicillin sensitive and methicillin resistant) with MIC value ranging between 3.25-12.5 µg/ml (Al-Zahrani, 2012).

No antioxidant activity was detected on hexane and dichloromethane extracts. The lowest antioxidant activity was achieved by water extract with IC_{50} values of 7.04±0.22 µg/ml. While ethyl acetate, methanol and acetone extracts achieved the best antioxidant activity compared to positive control of (+) catechin. In Fig (4.6) the results of lipase inhibitory activity clarified that the methanolic extract exhibited the strongest inhibitory effect with inhibition value 86.09±1.60 (acetone= 83.97±1.6, ethyl acetate= 79.65±0.04 and water= 79.37±1.2). The most active extracts on antioxidant activity and lipase inhibition were limited on methanol, acetone and ethyl acetate extracts. The HPLC chromatogram did not show clear differences between these three extracts. Therefore, methanoilc extract was selected for further research. Two compounds including gallic acid and methyl gallate were extracts by using elute (1) water/methanol (95:5 v/v) and elute (3) water/methanol (50:50 v/v) respectively (Fig 4.5).

It is a well known fact that the radical scavenging ability of any compound is a main focus to explain its protective function against oxidative stress. Keeping this in mind, the antioxidant activity of gallic acid and methyl gallate and other fractions were tested (Fig 4.7). The IC_{50} value for gallic acid was 1.51 µg/ml (8.90 µM), this data is similar to that reported in Yokozawa et al., (1998) and Lin Hsu et al., (2012) studies (IC_{50} value 8.14; 7.59 µM respectively). The IC_{50} value for methyl gallate was 0.70 µg/ml (3.81 µM), also this data is relatively similar to that reported in Pfundstein et al., (2010) and Lin Hsu et al., (2012) studies (IC_{50} value 4.28; 4.62 µM respectively). Additionally the fraction with Rt 27 has excellent activity similarly to methyl gallate (0.64 µg/ml). These finding are agree with Maldini et al., (2011) who indicated that *A. nilotica* pods, has a rich source of very strong antioxidant principles. However, Kaur et al., (2011) found that hydrogen donation and free radical scavenging potential of methyl

gallate was lower than standard antioxidant compound i.e. gallic acid and the lower effect was related to substitution of –OH group by –CH3 group . The study of antioxidant activity of isolated hydrolysable tannin, gallic acid and methyl gallate from *Osbeckia chinensis* by using two biological tests including; lipid peroxidation of rabbit erythrocyte membrane ghost induced by tert-Butyl hydroperoxide (t-BuOOH) and lipid peroxidation of rat liver microsome induced by ADP-Fe^{3+} - EDTA-Fe^{3+}-NADPH. All hydrolysable tannin and methyl gallate are exhibited stronger activities to reduce lipid peroxidation than gallic acid (Su J-D et al., 1988). Whang et al., (2005) investigated whether methyl gallate from medicinal plants protects human cells (Human umbilical vein endothelial cells (HUVECs)) from oxidative stress. Methyl gallate showed free radical scavenging effect at low concentration (0.02 mM) and cell protective effect against H_2O_2-mediated oxidative stress. Methyl gallate recovered viability of HUVECs damaged by H_2O_2-treatment, reduced the lipid peroxidation and decreased the internal ROS level elevated by H_2O_2-treatment. Free radical scavenging effect of methyl gallate was proven to be very high.

Gallic acid isolated form *A. nilotica* pods demonstrated good ability to inhibit the *p. acnes* lipase activity compared to methyl gallate and other fractions at the concentration of 125 µg/ml (Fig 4.8). This result was consistent with that reported by Oi et al. (2012), which stated that the gallic acid had good inhibitory activity against pancreatic lipase with IC_{50} value of 9.2 µg/ml. The compounds of gallic acid and methyl gallate isolated from *Galla Rhois* were evaluated for their ability to inhibit pancreatic lipase activity. These compounds found to be the inactive, with IC_{50} values more than 300 µg/ml (Kwon et al., 2013). Contradictory in the result could due to the different methods of *in vitro* evaluation of lipase inhibitory activity assay. In the last years

evidence supports a pivotal role for cellular inflammatory events at acne lesion development. The emphasis has moved from acne as a primarily hyperproliferative disorder of the sebaceous follicle to that of an inflammatory skin disorder (Holland and Jeremy, 2005). From previous reports about *A. nilotica* pods showed a good anti-inflammatory activity using *in vivo* model. Moreover methyl gallate was evaluated *in vivo* for its analgesic activities in mice and for NO and IL-6 production in RAW 264.7 cells. Methyl gallate inhibits LPS-induced NO and IL-6 production in RAW 264.7 cells. Consistent with these observations, the protein and mRNA expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were inhibited .Taken together, there is indication that methyl gallate has anti-inflammatory effects (Chae, 2010). So we speculate that may *A. nilotica* playing role for reducing acne inflammation.

Many studies indicated that the biological properties of *A. nilotica* due to tannin activity. Tannins have natural astringent properties and are used topically to treat acne (Philip, 2011). Witch hazel (*Hamamelis virginiana*) bark extract is commonly used as a household remedy by making a decoction from 5 to 10 g of herb in 1 cup (0.24 L) of water. Witch hazel is considered very safe to use topically and is Class 1 (McGuffin et al., 1997; Peirce et al., 1999). Hence, *A. nilotica* may contribute to treatment of acne as topical treatment after taking the consideration of safety in future.

In conclusion, gallic acid and methyl gallate are promising compounds that responsible for lipase inhibitory and antioxidant activity respectively. These two compounds could be useful to reduce the severity of acne. However, it should been noted that safety is a primary consideration for practical use in human.

Table 4.1. Antibacterial and antioxidants activities of *A. nilotica* extracts

Solvents	Yield%	MIC (mg/ml)	Antioxidant (IC_{50}µg/ml)
Hex	1.20 (0.36g/yellowish, oily)	-	ND
DCM	0.28 (0.1g/light green)	-	ND
EtoAc	2.78 (0.83g/brown)	2	0.73±0.01
$(CH3)_2CO$	17.48 (5.24g/dark brown)	2	1.11±0.02
Me OH	12.90 (3.88g/dark brown)	4	1.03±0.10
H_2O	5.91 (1.77g/ light brown)	-	7.04±0.22
IPMP		1	-
(+) Catechin		*	2.39

(-) No activity at the concentration of 4 mg/ml

(*) Not tested

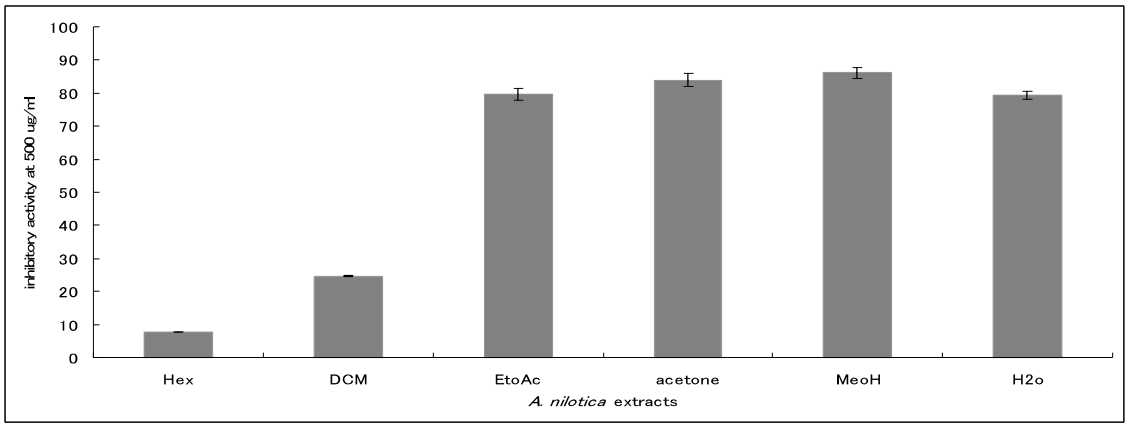


Fig 4.6. Lipase inhibitory activity of *A. nilotica* extracted by different solvents

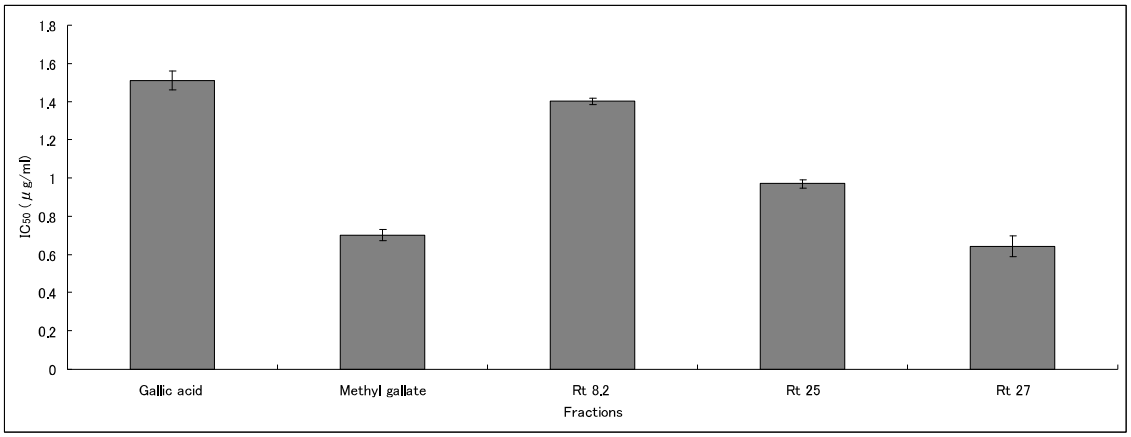


Fig 4.7. Antioxidant activity *of A. nilotica* methanolic extracts fractions

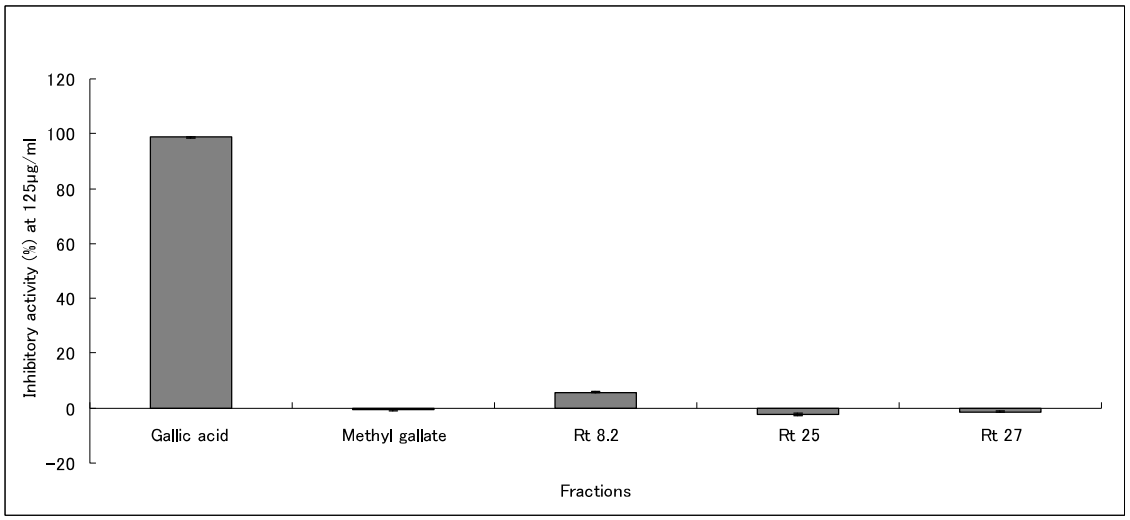
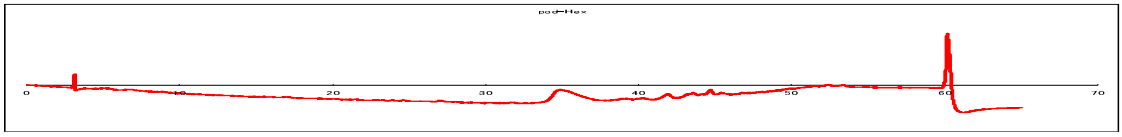


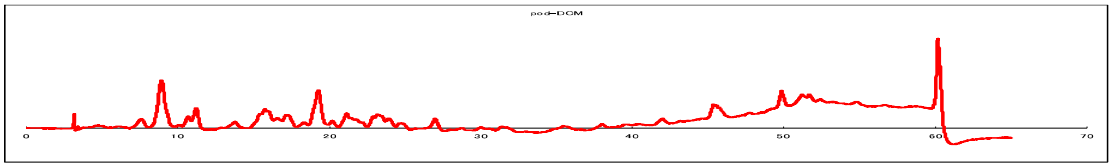
Fig 4.8. Lipase inhibitory activity of *A. nilotica* methanolic extracts fractions

Fig 4.9. HPLC chromatograms of *A. nilotica* pod extracted by different solvents

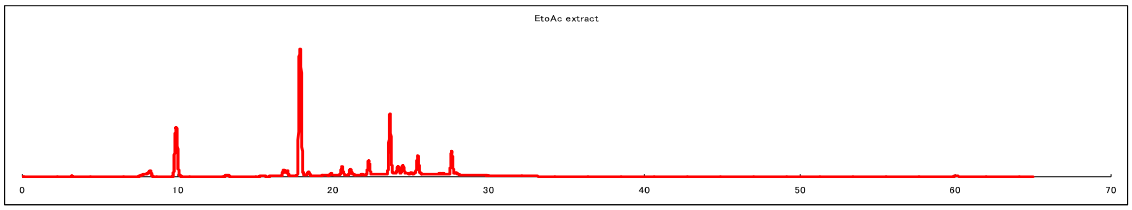
Hex extract



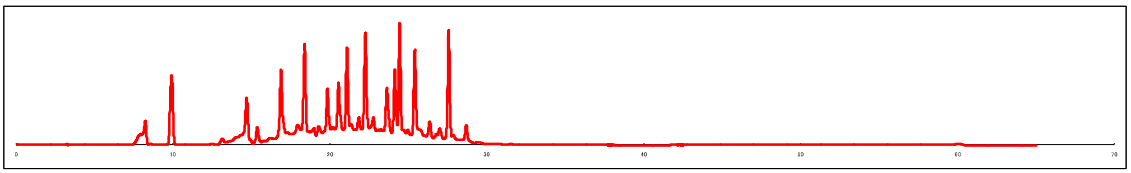
DCM extract



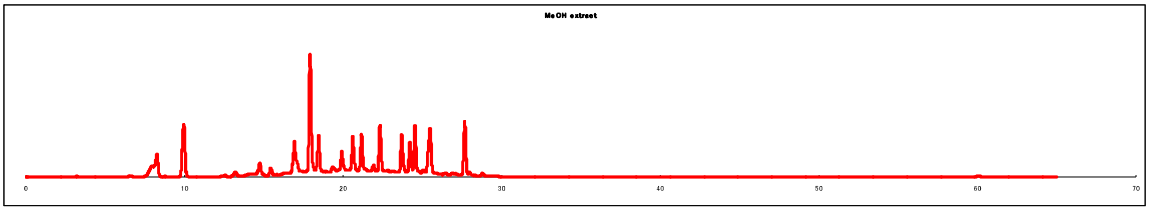
EtoAc extract



$(CH3)_2CO$ extract



MeOH extract



H_2O extract

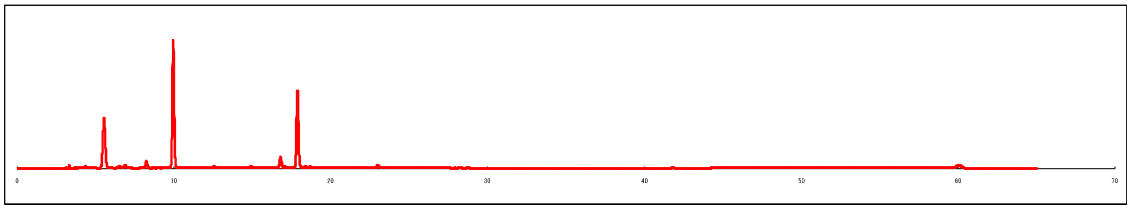
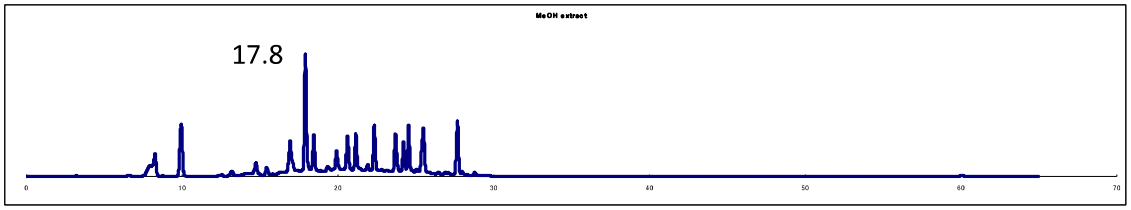
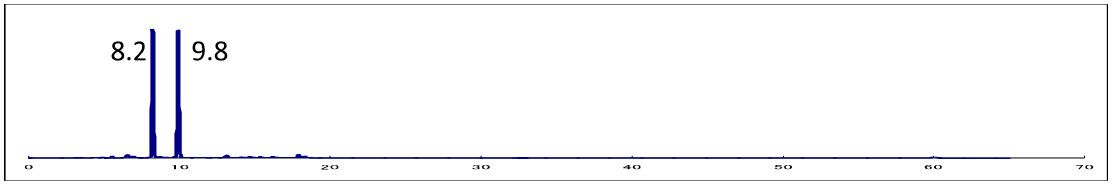


Fig 4.10. HPLC chromatograms of *A. nilotica* pod fractionated by MPLC

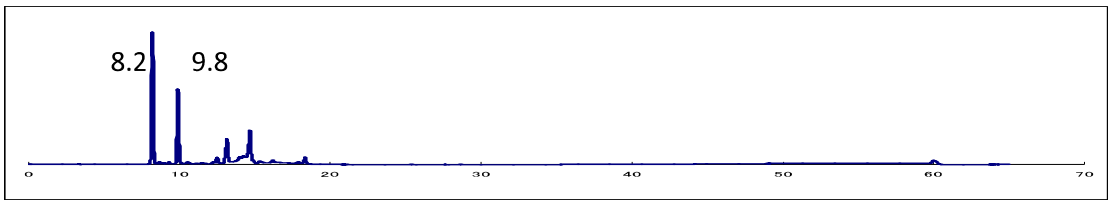
MeOH Extract



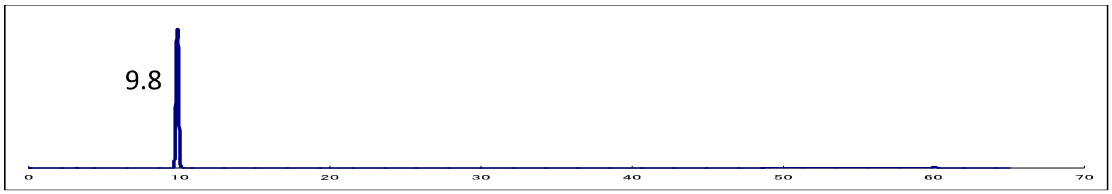
Elute (1) 95:5 H_2O:MeOH



Elute (2) 80:20 H_2O: MeOH

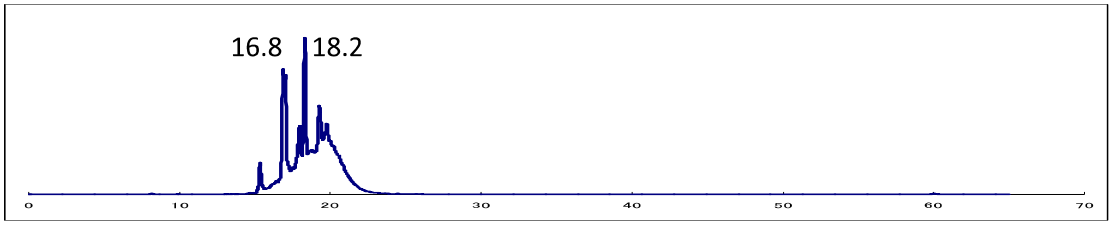


Gallic standard

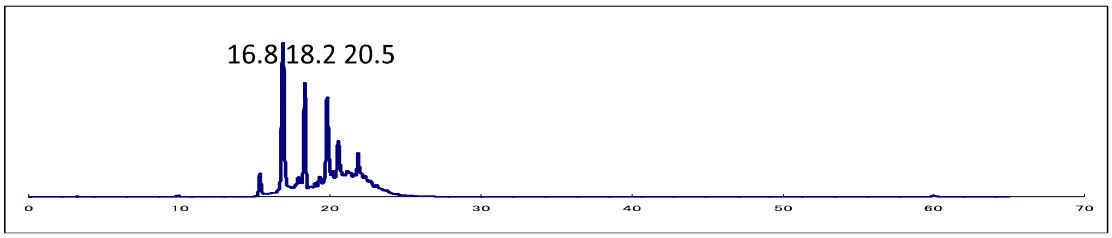


Elute (3) 50:50 MeoH: H_2O

Fr1



Fr2



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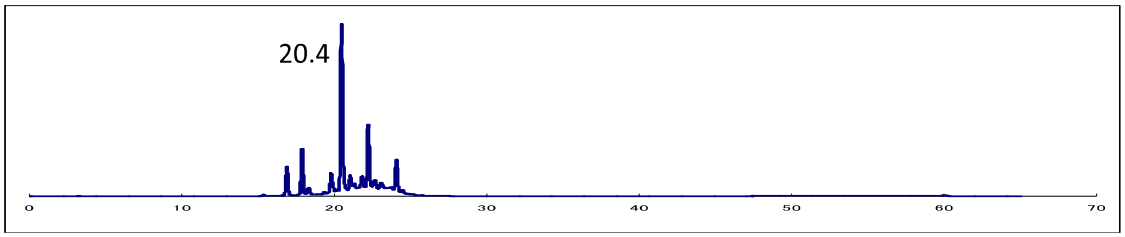
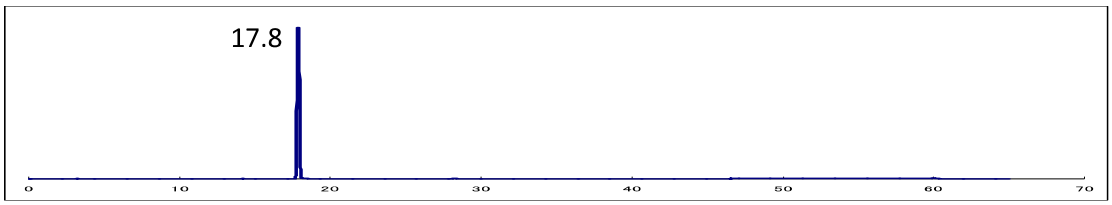


Methyl gallate standard



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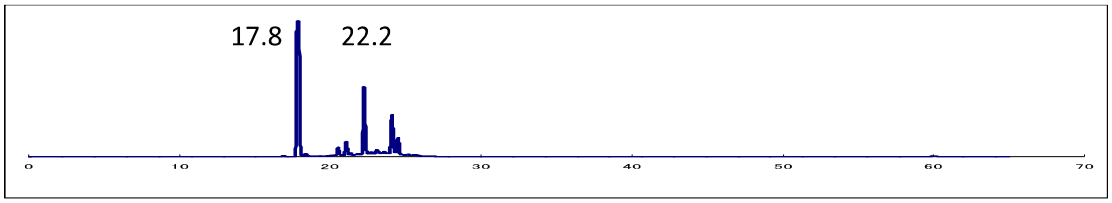

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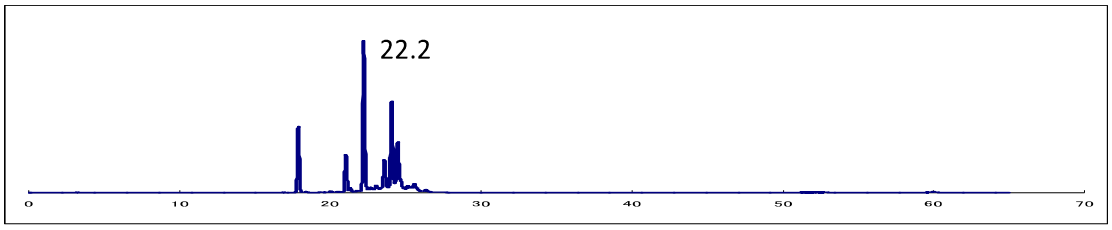

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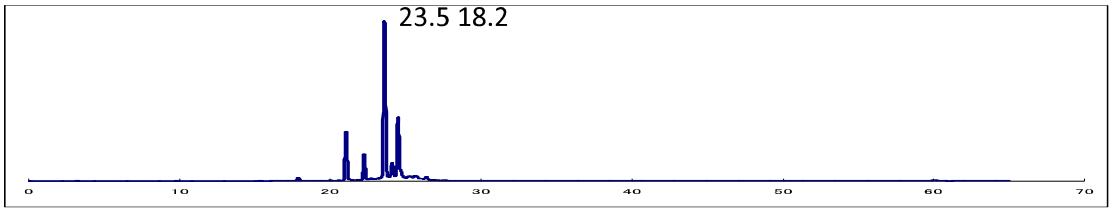

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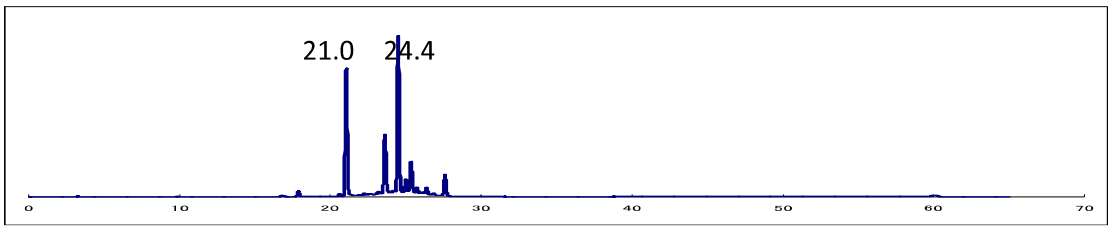
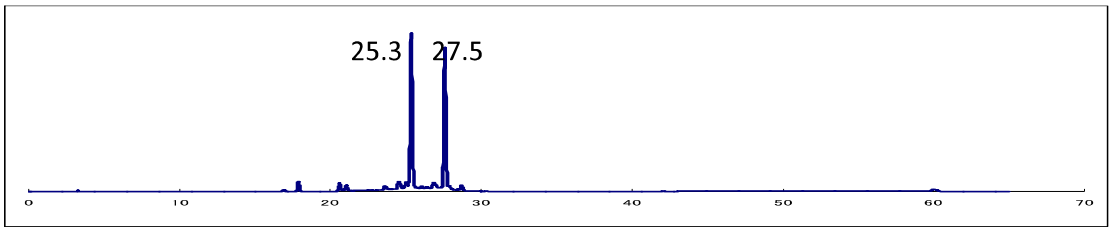


Fr8



General conclusion and perspectives

According to the World Health Organization (WHO, 2011) about 70–95% of the world's population in developing countries relies mainly on plants for their primary health care. Traditional medicine has not only gained popularity and approval, but it is sometimes the only system available in many rural areas. Furthermore, the use of medicinal plants to treat skin infections is very common in many rural areas. Medicinal plants used for dermatological purposes, both traditionally and in the cosmetic industry, are gaining more value, as many skincare products are now being supplemented with plant extracts. More specifically, in Sudan research on medicinal plants used for dermatological inflictions has not been given the attention it so rightly deserves. Acne is a common problem worldwide, subsequently the impact on the use of traditional medicines for the treatment of acne vulgaris can further pilot safer alternatives compared to the existing conventional treatments such as retinoids which are very aggressive and have severe side effects from photosensitivity reactions to teratogenic effects on unborn babies (Mabona and Van Vuuren, 2013).

This study has been set out to explore the concept of using some selected Sudanese medicinal plants for anti-acne activity. Work on anti-acne in Sudan is negligible. To the best of my knowledge no work has been done on anti-acne by using Sudanese medicinal plants. The anti-acne activity of theses selected Sudanese medicinal plants are based on their antimicrobial activity against *P. acne*, lipase inhibitory activity and antioxidant activity. The antimicrobial activity was determined by minimum inhibitory concentration (MIC) and minimum bactericidal activity (MBC), lipase inhibitory activity was measured by lipase assay kit and antioxidant activity was performed by DPPH radical scavenging. One hundred and four extracts included

methanol and 50% ethanol from forty plant species were used in this study. Among the tested plants, Combretaceae plant family seems to be the strongest antimicrobial agent against *P. acnes*. However methanol and 50% ethanol extracts of *T. laxiflora* wood exhibited excellent antibacterial activity. The 50% ethanol extracts of *A. precatorius* L. seed, *T. laxiflora* and methanol extract of *A. nilotica* (L) pods gave the best lipase inhibitory activity. The methanol extracts of *A. nilotica* (L) pods showed the outstanding DPPH radical scavenging activity. The best extract based on comprehensive activities was *T. laxiflora*.

T. laxiflora was selected for further purification by medium pressure liquid chromatography (MPLC), Sephadex LH-20 column and preparative HPLC. Four tannin related compounds were isolated and identified as ellagic acid, flavogallonic acid dilactone, terchebulin and gallic acid. As anti-acne agent terchebulin is prospective compound because of the great potentiality as antimicrobial, antioxidant agent and moderate lipase inhibitory activity. Gallic acid exhibited good lipase inhibitory activity.

The pods methanolic extract of *A. nilotica* studied has found to be an excellent source of antioxidants as they display good activity to scavenge free radical. They also possess a relatively good lipase inhibitor. Therefore, it was selected for further research. Two compounds well known were isolated; included methyl gallate and gallic acid. The methyl gallate was demonstrated the excellent antioxidant activity (IC_{50} 3.81 μM). Gallic acid again was responsible for potent lipase inhibitory activity.

From this study some compounds displayed a potentiality as anti-acne agent, however it should take in the consideration that *in vitro* results are not necessarily indicative of what could happen *in vivo*, therefore further studies are required. In order to optimize the use of prospective compounds the cytotoxicity, anti-inflammatory and

cellular antioxidants assays are necessary. Further work *in vivo* model should be taken in the future.

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