Sudan University of Science and Technology

College of Graduate Studies

Chromatographic Study of Activity of *Euphorbia Eaegyptiaca* extracts and their effects on germination of selected parasitic *Orobanchaceae* weeds

دراسة كروموتوغرافية لنشاطية مستخلصات الايفوربيا ايجيبتياكا وتأثيرها على إنبات أنواع مختارة من حشائش الاوروبانكي المتطفلة

A dissertation Submitted in Partial Fulfillment of the requirements of the degree of Master of Science in Chemistry

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October 2016

Sudan University of Science & Technology

College of Graduate Studies

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كلية الدراسات العليا

جامعة السودان للعلوم والتكنولوجيا

كلية الدراسات العليا

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Thesis title: Chromatographic Study of Activity of Euphorbia Faegyptiaca Extracts and Their Effects on Germination of Selected Parasitic Orobanchaceae weeds

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الآيـة

قال تعالي :

(فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ وَلَا تَعْجَلْ بِالْقُرْآنِ مِنْ قَبْلِ أَنْ يُقْضَى إِلَيْكَ وَحْيُهُ وَقُلْ رَبِّ زِدْنِي عِلْمًا).

صدق الله العظيم

سورة طه الآية (114)

Dedication

To my great parents Fatima and Hassan.

To my sisters, brothers, aunts, uncle.

Acknowledgement

All praises are due to Almighty Allah, the Omnipotent, the Omnipresent, the Most Gracious, and the Most Merciful, Alhamdulillah; I finished my dissertation with help and full support of my lord Allah, guidance of my supervisor, cooperation of friends and family.

I would like to express my deepest sincere gratitude to my supervisor Dr. Kamal Mohammed Saeed for guidance, patience and providing me with valuable advice to finalize this research. I want to express my deepest appreciation and sincere gratitude to Professor Abdel Gabbar Eltayeb Babiker for his kind and friendly supervision, valuable advice, guidance, personal assistance and unlimited support throughout this study period. I would like to thank Professor Yukihiro Sugimoto (Gradute School of Agricultural Science, Kope University, Japan) for his help with the analysis of Euphorbia stimulants, Special thanks to Dr. Rashida Mohammed Ahmed Abusin for her role in data analysis of experiments also I would like to thank Dr. Amani Hamad Eltayeb for her help, special thanks to my friends and colleagues at the *Striga* Research laboratory, Sudan University of Science and technology. I am indebted to the Japanese International Cooperation Agency (JICA) for this kind cooperation. Special thanks to Dr. same Jima for his support

Finally I would like to thank my father Hassan Ishag Algaddal and my mother Fatima Mohammed. They are always cheering me up and stood beside me supporting me financially and morally to carry my study to Master level, may Allah bless and grant them good health and wealth.

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List of Abreviation

ABA	**Abscisic Acid**
C°	**Degree Celsius**
CC	**Column Chromatography**
Cm	**Centimeter**
Fr	**Column Chromatography Fraction**
g	**Gram**
G%	**Germination Percentage**
GIA	**Germination Inducing Activity**
GFF	**Glass Fiber Filter paper**
GR24	**Synthetic Germination Stimulants**
h	**Hour**
l	**Litre**
µl	**Micro Litre**
LC-MS	**Liquid Chromatography - Mass Spectrometry**
PM	**Pico Molar**
RF	**Retention Factor**
RPM	**Recycle Per Mint**
TLC	**Thin Layer Chromatography**

Abstract

Extracts of *Euphorbia Eaegyptiaca* (fresh and dry shoots and latex) were assessed for ability to induce germination of seeds of selected root parasitic weeds (*Striga hermonthica* sorghum and millet strains, *Orobanche crenata* and *Phelipanche ramosa*). The seeds were then used to probe the chromatographic behavior of the active substances in an endeavour to develop cleanup protocols for the active compounds as prelude for further work on elucidation of structures and synthesis of analogues and/or mimics. *E. aegyptiaca* shoot (dry or fresh) and latex were extracted by a series of organic solvents, hexane, chloroform and ethylacetate, chosen on differential polarity. GR24 (a synthetic germination stimulant) and distilled water were used as positive and negative controls, respectively. The aqueous control induced negligible germination (0-1%) while GR24 effected 93% germination of *S. hermonthica*, irrespective of strain. Achieved seed germination results ranging of Hexane, chloroform, ethanol, butanol and ethylacetate extracts of dry *E. aegyptiaca* dry shoots induced 44%, 86%, 87%, 93% and 88% germination of *S. hermonthica* strain. The corresponding germination of the pearl millet congener were 35%, 87%, 59%, 21% and 67%. *P. ramosa* displayed 0, 33, 5, 5 and 13% germination when challenged the extracts as above. *O. crenata* on the other hand displayed no germination these results explain the polarity behavior of germination stimulants. Thin layer chromatography (TLC) using a mixture of ethyl acetate and hexane (7:3) as a developing solvent showed two active spots at Rf 0.14 and 0.29. Using equi-volume (1:1) of hexane: ethyl acetate did not affect further separation. On column chromatography two active fractions were identified. Further analysis using LC-MS showed no peak or a mass dsdssspectrum corresponding to 5-deoxystrigol, strigol, strigylacetate,

orobanchol, orobanchulacetate, sorgomol and sorgolactone. Lack of a ,m 7/mass spectrum corresponding to any of the aforementioned strigolactones (SLs) despite the detectable germination inducing activity suggest that the active ingredient(s) may be a novel SL, a non-SL compound or that the amount of the specific SL(s) is below the detection limits. It is noteworthy that bioassay using parasitic weed seeds are reported to be more than 100 times sensitive to SLs than instrumental analyses.

مستخلص البحث

تم تقييم قدرة مستخلص الايفوربيا الطازجة والجافة واللاتكس على حث إنبات بذور مختارة من الأعشاب الطفيلية الاتية: بودا الذرة الرفيعة وبودا الدخن، الحامول المتطفل على الطماطم و الهالوك المتطفل على الفول. ستخدمت هذه البذور للتحقق من السلوك الكروماتوغرافي للمواد الفعالة في مستخلصات الإيفوربيا لوضع بروتوكولات لتنقية المركبات النشطة كبداية للتعرف علي البنية لهذة المركبات النشطة وطرق تخليقها اوتخليق نظائرها. استخلصت الايفوربيا اجيتياكا (الجافة أو الطازج) واللاتكس باستخدام سلسلة من المذيبات العضوية، الهكسان، الكلوروفورم وخلات الإيثيل، أختيرت على الاستقطاب التفاضلي (إختلاف القطبية). أستخدم محفز الإنبات الإصطناعي (GR24) والماء المقطر كضوابط إيجابية وسلبية، على التوالي. أعطى الماء المقطر نسبة إنبات ضئيلة (0-1٪) كشاهد سلبي، في حين أعطى (GR24) نسبة إنبات 93% كشاهد ايجابي في بودا الذرة الرفيعة، بغض النظر عن السلالة لبذور الطفيل. حققت نتائج إنبات البذور في الهكسان، بيوتانول، الكلوروفورم, خلات الإيثيل والإيثانول في مستخلص الايفوربيا الجافة نسبة إنبات 44٪، 87٪، 86٪، 93٪ و88٪ لبذور طفيل بودا الذرة الرفيعة وكانت في المقابل إنبات بودا الدخن 35٪، 59٪، 77٪، 21٪ و 67٪. اما بذور الهالوك أوضحت نسبة إنبات 0، 33، 5، 5و 13٪ على النحو الوارد أعلاه. من ناحية أخرى لم توضح بذور الحامول أي استجابة أو إنبات لكافة المستخلصات.هذه النتائج تفسر السلوك القطبي لمحفزات الإنبات. إستخدمت كرموتوغرافية الطبقة الرقيقة(TLC) باستخدام خليط من مزيبات الهكسان وخلات الإيثيل (7:3) كطور متحرك أظهر منطقتين ذات نشاطية وقيمتا عامل الاستبقاء 0.14 ((Rfو0.29 .أستبدلت القطبية باستخدام حجم متساوي (1: 1) من الهكسان و خلات الإيثيل ولم تؤثر على الفصل. على كرموتوغرافيا العمود تم تحديد اثنين من التجزءات ذات النشاط الحيوي على إنبات البذور. أظهر المزيد من التحليل باستخدام كرموتوغرافيا السائل وطيف الكتلة (HPLC-MS) عدم وجود مطيافية مطابقة للتجزئتين مع اي من مخفزات الإنبات القياسية المعروفة وهي5- deoxystrigol, strigol, strigylacetate, orobanchol) (orobanchulacetate, sorgomol and sorgolactone. لأي منstrigolactones (SLS)السابق ذكرة مما يشيرهذا إلى أن العنصر النشط قد يكون نوع جديد من مخفزات الإنبات (SL) أوهو مركب غير (SLS) أو أن كمية المحفز كانت أقل من الحدود الدنيا للمقدرأو الكاشف. ومن الجدير بالذكر أن التحليل الحيوي باستخدام بذور الحشائش الطفيلية له حساسية أكثر بي 100مرة ضعف حساسية التحليل الآلي.

CHAPTER ONE

INTRODUCTION

Sorghum□*Sorghum bicolor* (L.*) Moench*□, millet [*Pennisetum glaucum* (L.)], maize [*Zea mays*L.] and cowpeas [*Vigna unguiculata (*L.*) walp*], the major hosts, of *Striga* spp, are produced in different geographical regions in the world under both irrigated and rain-fed conditions (Hamdoun and Babiker, 1978). Hence the growing areas of these crops comprise wide array of soil types and climatic conditions. These crops constitute the most important staples in sub-Saharan Africa where their production is constrained by both biotic and a biotic factors.

The most important biotic factors limiting production mbrace diseases, insect pests and the root parasitic weeds *Striga* spp(Parker and Riches, 1993). The major a biotic factors affecting production are drought, above-normal temperature and below-normal rainfall, wet weather at harvest, excessive rain and lodging (Assefa *et al.*, 2010). However of all factors limiting yield *Striga hermonthica* (Del.) *Benth* on sorghum, maize and millet and *Striga gesneridios (Willd.) Vatke, Oesterr. Bot. Z.).* On cowpea are the most important.

Root parasitic weeds are spreading at a rapid rate nearly 100 million hectares of the African savannah are infested annually with *Striga* (Ejeta 2007; Rich and Ejeta, 2008). Root parasitic plants are socioeconomic problems that have forced resource poor farmers to abandon their land (Atera *et al.*, 2012). *Striga* damage to crops is often severe because of its remarkable be witching effects on the crops it invades. Losses in grain yield due to *Striga* infestation in Africa were estimated to be about 40% when averaged across the continent (Lakoge *et al.*, 1991). The magnitude of the loss depends on the parasite seed bank size, susceptibility of the crop, climatic conditions and nature of soil (Lagoke *et al.* 1991; Sallè *et*

al.,1987). Grain yield losses may reach 100% in susceptible cultivars under high infestation level and drought conditions (Haussmann *et al.,* 2000).

The *Striga* problem undermines the struggle to attain food security and economic growth in the continent. The parasite is a severe problem for millions of smallholders throughout the semi-arid areas of Africa and part of Asia (Parker and Riches, 1993).

Striga, poor farmer's problem, is a direct result of demographic and economic pressures in farming communities. The limited crop choice inferred by drought, poor soil fertility and intermittent rainfall make a near perfect ecological overlap between areas infested by the parasite, poor farmers and hunger prevalence. Furthermore the regions are often characterized by low rainfall and degraded, infertile soils (Gressel *et al.,* 2004).

Striga lifecycle is strongly cued to that of it host. In nature *Striga* seeds only germinate in response to chemical stimulants exuded by host and non-host plant roots. Following germination, the radicle elongates and in response to a second chemical signal, a haustorium is formed (Hood *et al.,* 1998). The haustorium, a physiological bridge between the host and the parasite, attaches, penetrates the root and establishes connection with the host xylem. Following connection with the host xylem the parasite develops and stays subterranean for 6-8 weeks before emerging over the ground .The parasite dose most of its damage while subterranean, where it is not amenable to conventional control measures (Yoder, 1999).

Germination is the first step in the life cycle of the parasite. Induction of the parasite seeds to germinate in absence of hosts or away from host root "Suicidal germination" has been thought of by several workers as means for depleting seed reserve. Work with several *Euphorbia* species showed that their shoot extract induced high germination of the parasite seeds

(Ibrahim *et al.*, 1985). *EuphorbiaEaegyptiaca* is not a conventional host of *S. hermonthica, O. crenata or p. ramosa.* Identification of the stimulants may offer a new chemistry which could be used as a lead for synthesis of new and more effective germination stimulants. The present study was therefore designed to i) assess the germination inducing activity of *E.E.aegyptiaca,* latex, and fresh and dry shoot extracts and ii) study chromatographic behavior of germination stimulant in *E. aegyptiaca* latex and shoot extracts.

CHAPTER TWO
LITEATURE REVIEW

2.1. Chemistry of natural products

Plants produce an enormous variety of natural products with highly diverse structures. These products are commonly termed "secondary metabolites" in contrast to the "primary metabolites" which are essential for plant growth and development. Secondary metabolites were formerly regarded as "waste products" without physiological function for the plant. With the emergence of the field of chemical ecology about 30 years ago, it became evident, however, that these natural products fulfill important functions in the interaction between plants and their biotic and a biotic environments (Springob and Kutchan, 2009). The secondary metabolites serve as defense compounds against herbivores and pathogens, as flower pigments that attract pollinators, or as hormones or signal molecules. In addition to their physiological function in plants, natural products also have a strong impact on human culture and have been used throughout human history as condiments, pigments, and pharmaceuticals. The chemistry of natural products is essentially the chemistry of substances found in living organisms (Hartmuth and Sharpless, 2003).This definition includes many compounds such as carbohydrates, proteins, lipids and nucleic acids, all are essential for living organisms. These compounds are primary metabolites. There are other organic compounds produced in nature which are not primary metabolites and they are designated as `secondary metabolites. Organic chemists have focused their interest on secondary metabolites, particularly those isolated from plants. During the nineteenth century these products constituted the main source of organic chemistry (Polo, 1981). The interest in these substances exists for a variety of reasons, ranging from the particular applications of such

compounds in daily life and to the scientific challenges presented by them. Thus people have used natural products to alleviate pain, to cure diseases, to provide colourful dyes for their bodies and clothing and to flavour food (Robert *et al.,* 1979). The use of plants to prevent and cure diseases goes far back in history of man, and natural products are now of scientific interest and commercially important because they are produced continuously, albeit often in small amounts, by green plants through photosynthesis and subsequent specific pathways from inorganic materials using only solar energy (Shafik *et al.,* 1981). Determination of structures by various analytical methods has also been greatly developed (Mahato *et al.*, 1998).

2.1.1. Classification of Natural products

There are various methods for classifying naturally occurring compounds. They may be classified according to the source from which they are obtained or they may be classified according to their physiological effects, e.g. antibiotics. However, the majority of chemists prefer to classify natural products according to their structure (Hartmuth and Sharpless, 2003). Thus natural products were classified into the following groups: terpenes, steroids, alkaloids, flavonoids, xanthones, coumarins, quinines (Alan and Putnam, 1985).This classification, however is overlapping e.g. an alkaloid with 20 carbons may be classified as diterpene (C_{20}), vitamin A may also be classified as a diterpene. Since flavonoids, quinines, coumarins and xanthones and some vitamins and antibiotic have phenolic properties; the natural products may be divided into a smaller number of groups including i) Terpenes and steroids, ii) alkaloids, acetogenins (plant phenolic compounds) and iii) carbohydrates.

2.2. Plant hormones

Thimann (1948) designated the plant hormones by the term 'phytohormones' in order to distinguish them from animal hormones. He defined a phytohormone as "an organic compound produced naturally in higher plants, controlling growth or other physiological functions at a site remote from its place of production and active in minute amounts."

A definition of plant hormones with still wider scope has been given by Johannes van Overbeek (1950). According to him, the plant hormones are defined as "organic compounds which regulate plant physiological process regardless of whether these compounds are naturally occurring and/or synthetic; stimulating and/or inhibitory; local activators or substances which act at a distance from the place where they are formed."

The migratory nature of hormones has been specifically emphasized by Meirion Thomas (1956) who stated, that "All hormones are migratory correlating substances or correlators which play an essential part in the integration of plant behaviour."

Three types of plant hormones are usually recognized. These are auxins, gibberellins and cytokinins. These were discovered in the early decades of the twentieth century, in 1930's and in 1960's respectively. Naturally, the knowledge accumulated on auxins and gibberellins is far greater than that gathered for cytokinins (Chauhan, 2008).

2.2.1. Classes of plant hormones

In general, it is accepted that there are five major classes of plant hormones, which are made up of many different chemicals that vary in structure from one plant to another. The chemicals are each grouped together into one of these classes based on their structural similarities and on their effects on plant physiology. Other plant hormones and growth regulators are not easily grouped into these classes; they exist naturally or are synthesized by humans or other organisms, including chemicals that

inhibit plant growth or interrupt the physiological processes within plants. Each class has positive as well as inhibitory functions, and most often work in tandem with each other, with varying ratios of one or more interplaying to affect growth regulation (*Weier et al.,1979*).

The five major classes are: i) abscisic acid, ii) auxins, iii) cytokinins, iv) ethylene and v) gibberellins, other known hormones are:brassinosteroids, salicylic acid, jasmonates,plant peptide hormones division, polyamines, nitric oxide (NO) and Strigolactones(Bosch andMüller (2013).

2.3. Allelopathy Research in Agriculture

Allelopathy which comes from the two Greek words "allele" and "pathy" means "reciprocal suffering of two organisms"; thenceforth, in the literature the term usually implies plant-plant interaction which occur through biochemical effects (Putnam and Tang, 1986).

The term allelopathy was coined by Molisch in 1937. Presently, the term generally refers to the detrimental effects of higher plants of one species (the donor) on germination, growth, or development of plants of another species (the recipient). Allelopathy can be separated from other mechanisms of plant interference because the detrimental effect is exerted though release of chemical inhibitors (allelochemicals) by the donor species. Microbes associated with the higher plants may also play a role in production or release of allelochemicals through decomposition of plants residues (Alan and Putnam, 1985).

Allelopathy is included among a higher-level order of chemical ecology involving interactions among many different organisms. Whittaker and feeny (1971) have defined interspecies allelochemicals effects and classified allelochemicals on the basis of whether the adaptive advantage is gained by the donor or recipient. Allomones, which give adaptive advantage to the producer inductants, counteractants, and attractants. Allelopathic chemicals may be classified as a suppressant some inhibitors

from plants may also induce intraspecific effects (autotoxicity) (Alan and Putnam, 1985).

2.3.1. Natural products Identified as allelopathic Agents

Allelochemicals from plants and their associated microbes represent a myriad of chemical compounds from the extremely simple gases and aliphatic compounds to complex polycyclic aromatic compounds (Alan and Putnam, 1985). The compounds implicated in allelopathy have been divided into chemical classes by recent reviewers, they can be arbitrarily classed as i) hydrocarbon, ii) organic acids and aldehydes, iii) aromatic acid, iv) simple unsaturated lactones v) coumarins, vi) flavonoids, tannins, vii) alkaloids, viii) terpenoids and steroids and (ix) miscellaneous and unknowns. Although many of these compounds are secondary products of plants metabolism, several are also degradation products, which occur in the presence of microbial enzymes (Alan and Putnam, 1985).

2.4. Chemistry of host- parasite interaction an emerging branch in allelopathy

Enhancement of *Striga* seed germination by host root exudates is an allelopathic effect (Rice, 1984).Parasitic weeds represent an emerging branch of research in allelopathy. Parasitic plants constitute an economical threat for many important crops, and the tandem host-parasite is one of the systems where chemically mediated plant recognition is better proved (Galindo *et al.*, 2004). Germination in parasitic plant only takes place when the seed detects the presence in soil of specific chemical signals from their hosts. The family Orobanchaceae has received much attention due to economical reasons, as it includes the most devastating root parasitic weeds, witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.). Up to date, only few compounds have been isolated and characterized as seed germination signals from natural hosts of these species. These compounds belong to tow different chemical

families, quinines (e.g., sorgoleone) and sesquiterpenes (e.g., sorgolactone). However, compounds isolated from non-host plants and organism, and synthetic derivatives as well, have also induced germination responses to different extents. A chemical model to explain the interaction between the inductor and the active site in the parasite has been proposed, but not all active compounds fit into this model, thus suggesting than more than one mechanism or site of action is involved. The study of host-parasite chemical interactions is a relatively new field of research in allelopathy that is receiving increasing attention for economical and scientific reasons. The existence of parasitic plants has been reported since ancient times. Plants belonging to family *Orobachaceae* were described by dioscorides and the genus *Orobanche* was portrayed by Linnaeus in 1793 (Galindo *et al.*, 2004). Over 4000 species of parasitic plants grouped in 20 families have been described so far (Musselman, 1987). There are four families of special importance because of their adverse impact on different crops, namely *Orobanchaceae*, *Cuscutaceae, Viscaceae* and *Loranthaceae*. Among them, weeds belonging to the family *Orobanchaceae* are important economical threats to crops such as legumes, several gramineae, tomato, sunflower, and tobacco (Galindo *et al.*, 2004) (Appendix 1). However, parasitic weeds control techniques have not been studied until recently, and proper control methods are not available yet.

Parasitic weeds can be broadly divided into hemiparasites and holoparasites, according to the presence or absence of chlorophylls. The holoparasites, being a chlorophyllus, fully depend on their hosts for mineral nutrients and carbohydrates to complete their life cycle, as they are not able to fix carbon through photosynthesis. The hemiparasites are chlorophyllus and only obtain minerals, water and carbohydrates from their hosts and their parasitism can be facultative (Nickrent and

Musselman 2004). Tow well-differentiated phases can be established in the life cycle of parasitic Orobanchaceae the non- dependent phase and the dependent phase. The former constitutes germination, haustorium formation and attachment, whereas the latter spans penetration and subsequent stages in the lifcycle.

2.4.1. Weedy root parasites

Among the parasitic angiosperms, witchweeds (*Striga* spp.) and broomrapes (*Orobanche* and *Phelipanche* spp.) in the family Orobanchaceae are the most devastating weeds that parasitize roots of host plants (Parker and Riches 1993, Joel *et al.*, 2007, Parker, 2009), (Yoneyama *et al.*, 2010) and(Mitsumasu*et al.*, 2015). *Striga* species are hemiparasites as they have functional chloroplasts. However, their photosynthesis cannot support their survival without connecting to host roots and thus they are obligate parasites (Yoneyama *et al.*, 2010). *Striga* spp. are serious weeds of important food crops including sorghum, maize, pearl millet and rice [*Oryza sativa (*L.*)*] in sub-Saharan Africa, the Middle East and Asia (Musselman, 1980; Rodenburg *et al.*, 2006; Scholes and Press 2008). Their effects are severe, and complete loss of harvest is not uncommon in heavily infested areas. *Orobanche* and *Phelipanche* species are Chlorophyll-lacking holoparasites, which attack economically important dicotyledonous crops, including tomato [*Solanum lycopersicum*L.], tobacco*[Nicotiana tabacum* L.], carrot [*Daucus carota subsp. Sativus (Hoffm.) Schübl.&G. Martens.*], clover [*Trifolium* spp L.], cucumber[*Cucumis sativusL.*], sunflower [*Helianthus annus* (L.)] and legumes (Yoneyama *et al.*, 2010). Seeds of the root parasites are extremely small (0.2–0.4 mm) and composed of a relatively small number of cells (Joel *et al,*. 1995). Although non-parasitic plants, in general, produce several hundred to several thousand seeds, a single plant of *Striga* or *Orobanche* usually produce up to half a million seeds which remain

viable in the soil for many years (Yoneyama *et al.*, 2010). Large numbers of long-lived seeds ensure that these root parasites adapt to environmental changes, host resistance, etc., and consequently make them difficult to control (Joel *et al.,* 1995, Joel *et al.*, 2007).

2.4.2. Seed germination and dormancy

2.4.2.1. Germination

By definition, germination incorporates those events that commence with the uptake of water by the quiescent dry seed and terminates with the elongation of the embryonic axis (Bewley and Black, 1994). The visible sign that germination is complete is usually the penetration of the structures surrounding the embryo by the radicle; the result is often called visible germination. Subsequent events, including the mobilization of the major storage reserves, are associated with growth of the seedling. Virtually all of the cellular and metabolic events that are known to occur before the completion of germination of non-dormant seeds also occur in imbibed dormant seeds; indeed, the metabolic activities of the latter are frequently only subtly different from those of the former. Hence, a dormant seed may achieve virtually all of the metabolic steps required to complete germination, yet for some unknown reason, the embryonic axis (i.e., the radicle) fails to elongate (Bewley, 1997).

2.4.2.2. Dormancy

Dormancy is an innate seed property that defines the environmental conditions in which the seed is able to germinate" (Finch-Savage and Leubner-Metzger, 2006). According to this definition, dormancy is not only associated with the absence of germination, but it is a seed characteristic that determines the conditions required for germination. This definition better fits the results of many studies on seed germination and dormancy. Many of these deal with seeds possessing different levels of non-deep dormancy and dormancy is evaluated according to the ability of the seed to germinate under different conditions, for instance: light or dark, different temperatures, different water potentials or different external ABA concentrations. Seed dormancy could be considered simply as a block to the completion of germination of an intact viable seed under favourable conditions (Hilhorst, 1995; Bewley, 1997).

Dormancy has evolved differently across species through adaptation to the prevailing environment, so that germination occurs when conditions for establishing a new plant generation are likely to be suitable (Hilhorst, 1995; Bewley,1997a). Therefore, a diverse range of blocks (dormancy mechanisms) have evolved, in keeping with the diversity of climates and habitats in which they operate. A more sophisticated and experimentally useful definition of dormancy has recently been proposed by Baskin and Baskin (2004). They proposed that a dormant seed does not have the capacity to germinate in a specified period of time under any combination of normal physical environmental factors that are otherwise favorable for its germination.

Dormancy should not just be associated with the absence of germination; rather, it is a characteristic of the seed that determines the conditions required for germination (William *et al.*, 2006).

2.4.3. Lifecycle of *Striga* and *Orobanche*

The life cycles of *Striga* and *Orobanche* are very similar, and a number of mechanisms ensure the co-ordination of the parasites' life cycles to that of their hosts (Fig. 2.1). *Striga* or *Orobanche* seeds germinate after a pre-incubation period of moist and suitable temperatures (Phase I), and only when they perceive host-derived chemicals, termed 'germination stimulants', released from host roots (Phase II), ensuring that only seeds within the host rhizosphere germinate (Joel *et al.* 1995, Joel *et al.* 2007). The parasite seedling radicle grows only a few millimeters and must reach a host root, within a few days, before exhausting the resources in the tiny seed. Upon contact with the host root (Phase III), the radicle develops a specialized organ, the haustorium, which adheres to the root, penetrates the epidermis and cortex tissues of the root (Phase IV) and ultimately establishes connections to the host vascular system. Through this connection the parasite draws water and its nutritional needs from the host (Phase V). The parasite tubercles grow underground for several weeks (*Striga* spp.) to several months (*Orobanche* spp.) (Phase VI) and then produce aboveground flowering shoots (Phase VII). So far, three different types of compounds have been identified as germination stimulants for root parasitic plants; dihydroquinones, sesquiterpene lactones and strigolactones (SLs) (Bouwmeester *et al.* 2003). Among them, SLs are the most potent stimulants, inducing germination at ≤ 10 pM (Kim *et al.* 2010), and also acting as host recognition signals for symbiotic arbuscular mycorrhizal fungi (Akiyama *et al.* 2005 , Besserer *et al.* 2006). In addition to these functions as rhizosphere signaling chemicals, SLs also function as plant hormones inhibiting shoot branching (Gomez-Roldan *et al.* 2008, Umehara *et al.* 2008 , Xie *et al.* 2010).

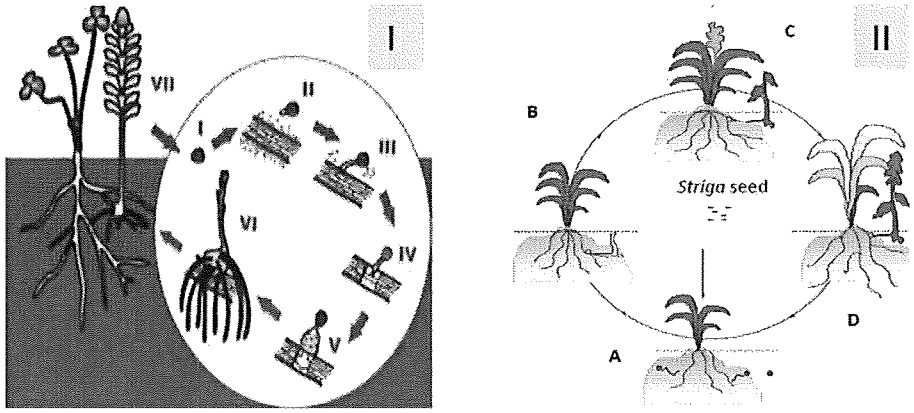


Fig. 2.1: The life cycles of the root parasitic plants *Orobanche* minor I and *Striga II*. Adapted from Yoneyama, *et al.*, (2010) and Nweze *et al.*, 2015, respectively.

2.4.4. Control methods

Many different control strategies have been developed to reduce the dramatic effects of root parasitic plants on crops (Babiker, 2007). In general, control strategies against root parasitic plants may be classified into two different types i) control methods targeting pre attachment and ii) control methods targeting post-attachment life cycle stages. These strategies include both traditional and sophisticated techniques. Techniques in use include hand weeding (Parker and Riches, 1993), crop rotation(Babiker *et al.*, 2007), suicidal germination (Eplee, 1975), improving soil fertility, soil fumigation and solarization (Sahile *et al.*, 2005), biological control(Klein and Kroschel, 2002), selective herbicides and breeding for resistance (Joel *et al.*, 2007; Scholes and Press, 2008).

Despite the high potential of some of those solutions so far no single option has shown to be both sufficiently effective and durable as well as economically and practically applicable for low-input farming systems (Joel, 2000). Because the plant parasites exert much of the damage to host crops during the early phases of attachment, control approaches should target the initial steps in the host– parasite interaction,

of which germination is the first (López-Ráez *et al.*, 2009). In this thesis emphasis will be laid on germination stimulants and suicidal germination. Other methods of control are well reviewed by Parker and Riches (1993). Bouwmeester *et al.*, (2003) suggested that the infection of the parasitic plant can be reduced by lowering strigolactone production in hosts. Some consequences of management approaches using knowledge of germination stimulants are discussed below.

2.4.4.1. Suicidal germination

Suicidal germination is an alternative approach to control *Striga* infestation using specific chemicals that induce *Striga* germination in absence of or away from the host roots. It could be achieved by synthetic germination stimulants and by catch and/or trap crops. The latter in monoculture or as intercrops (Chittapur *et al.*, 2001). Germination stimulants when applied to the soil in absence of a suitable host induce suicidal germination which leads eventually to depletion of the seed reserve.

Biosynthesis of ethylene, which is a plant growth regulator, in *Striga* seeds is induced by germination stimulants, natural and synthetics (Babiker *et al.*, 1993). Ethylene was the main stay of the US *Striga* eradication programme. The gas, under high pressure, was injected into soil (Eplee and Langston, 1991). However ethylene is a pressurized gas, flammable and requires specialized storage and application equipment for soil injection. Thus direct usage of ethylene is hazardous and it is not suitable for subsistent African farmers (Eplee 1975; Berner *et al.*, 1999). Flowing isolation of strigol several strigol analogues were synthesized and proved to be as effective as strigol in laboratory tests (Johnson *et al.*, 1981; Ibrahim *et al.*, 1985). Employment of strigol analogues as suicidal germination agent was also reported by Eplee and Norris, (1987). Applying GR 24 and GR 7 to *Striga*-infested soil resulted in a

considerable reduction (up to 50%) in the seed population. However, the compounds displayed complete loss of activity within one day when incubated in heavy alkaline soils (Babiker *et al.*, 1987). Further development was not pursued because of untimely decomposition of the compounds (Eplee and Norris, 1987). Suicidal germination could be achieved by employing catch or trap cropping. However, a catch or a trap crop should be a species that copiously produces germination stimulants in order to induce massive suicidal germination. Catch crop practice employs planting *Striga* host at high density and allowing it to grow for 5 to 6 weeks prior to ploughing (Timson, 1945). Two catch crops in a single season were reported to reduce *Striga* infestation to tolerable levels (Bebawi, 1987; Parker and Riches, 1993). However, this practice is not acceptable to farmers because of high cost and no immediate returns. Moreover, in the semi-arid areas, where *Striga* thrives best, seasons are often too short to support more than one crop (Parker and Riches, 1993). Trap crops are crops that produce germination stimulants, but are not susceptible to *Striga* attack. Rotation with trap crops was reported to be less effective in reducing *Striga* seed bank in the dry and less humid East Africa. Work in Eastern Kenya showed that 4 years of continuous cropping with cowpea or cotton did not reduce *Striga* infestation below damaging levels (Ransom, 1999).

2.4.5. Strigolactones

Strigolactones are important signalling molecules that were first described as germination stimulants for the seeds of parasitic plants of the genera *Striga* and *Orobanche* (Cook *et al.*, 1972; Bouwmeester *et al.,* 2003). Later, they were also described as hyphal branching factors for germinating spores of the symbiotic arbuscular mycorrhizal (AM) fungi (Akiyama *et al.*, 2005). Therefore, strigolactones play a dual and important role in the rhizosphere as host detection signals for AM fungi

and root parasitic plants (Akiyama *et al.*, (2005), Harrison, (2005), Paszkowski, (2006) and Bouwmeester *et al.*, 2007). In addition to their important role as rhizosphere signalling molecules, it has recently beendemonstrated that strigolactones also act as a new hormone class that inhibits shoot branching in plants and hence regulates aboveground plant architecture (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Strigolactones have been detected in the root exudates of a wide range of monocotyledonous and dicotyledonous plant species. The strigolactones discovered so far all have a similar basic chemical structure with variations in substituents and/or stereochemistry suggesting that they are all derived from the same biosynthetic pathway (Bouwmeester *et al.*, 2007; Yoneyama *et al.*, 2008).

2.6. Euphorbia (spp.) as allelopathic plants

Euphorbiaceae is a largest family among Anthophyta, with 300 genera and 5000 species distributed all over the world (Uzair *et al.*, 2009).The members of this family are well known for the production of a large number of secondary metabolites.The presence of these significant chemicals in the members of the *Euphorbiaceae* has been demonstrated by many phytochemical studies (Salatino *et al.*, 2007). All species of Euphorbia contain white latex, which is rich in secondary metabolites possessing different types of activities. Some of the extracts of Euphorbia species are registered drugs, for instance Euphorbium (resiniferatoxin), from the latex of *Euphorbia resinifera* A.Berger, marketed as 'Complexe Lehning Euphorbium N 88', is used as a nasal spray against chronic nasal discharge, dry and inflamed different types of secondary metabolites have already been isolated from some of the plant species of the family *Euphorbiaceae* (Rehmana *et al.*, 2014).

Members of the genusEuphorbia are known to contain substances which are inhibitory to seed germination and seedling growth as well as to

bacteria. The inhibitory action has been attributed to the presence of large amounts of phenolic compounds (Adedapo *et al.*, 2004 and Rice, 1965; 1974). However, the presence of lactone-forming acids in this genus has also been reported (Nordal and Benson, 1969; Kringstad, 1980). Both phenolic compounds and lactone-forming acids are of interest from the viewpoint of *Striga* seed germination. Phenolic compounds are known as inhibitors of *Striga* seed germination (Lakshim and Jayachandra, 1979), while some lactone-forming acids possess stimulatory properties (Long, 1955). More-over, phenolic compounds and flavonoids are known as haustorial inhibitors (Steffens *et al*,. 1982).Euphorbia species are of wide occurrence in tropical regions and their distribution is not limited by climatic or soil conditions (Holm *et al*,. 1977). For this reasons and previous reports on *Striga* germination inducing activity of aqueous extracts of several Euphorbia species (Ibrahim *et al.*, 1985) it was decided to confirm previous findings, extended the study on germination inducing activity to other parasitic *Orobanchaceae* and develop a cleanup procedure for the germination stimulant(s) in *E. aegyptiaca* in an endeavour to elucidate it/their structure(s).

CHAPTER THREE

MATERIALS AND METHODS

3.1. General

A series of laboratory experiments was undertaken at the College of Agricultural Studies, Sudan University of Science and Technology at Shambat with the primary objectives of i) assessing ability of *E. aegyptiaca* shoot and latex extracts to induce germination of seeds of the root parasitic weeds *S. hermnthica* (sorghum and millet strains), *P. ramosa* and *O. crenata* and ii) developing a protocol for isolation, purification and identification of *Striga* germination stimulant(s) in the extracts.

3.1.1. Material

3.1.1.1. Plant materials:

S. hermonthica seeds, sorghum and pearl millet strain, were collected in 2012 from the Gedarif and Kordofan state, respectively. *O. crenata* and *P. ramosa* seeds were collected in 2012 from fields in Shambat grown to faba beans [*Vicia faba* L] and tomato [*Solanum lycopersicum*L.], respectively. *E. aegyptiaca* was collected from the college of Agriculture studies turfs loans.

3.1.1.2. The synthetic *Striga* germination stimulant

[The synthetic *Striga* germination stimulant GR24 (Fig.3.1)] was obtained from professor Zewannenberg Radpound University of Nimjgen The Netherlands.

Molecular Formula: $C_{17}H_{14}O_5$

Molecular Weight: 298.29

IUPACName:(3E,3aR,8bS)-3-[[(2S)-4-methyl-5-oxo-2H-furan-2-yl]oxymethylidene]-4,8b-dihydro-3aH-indeno[1,2-b]furan-2-one.

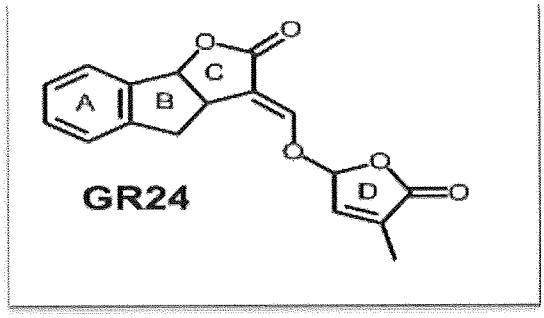


Fig.3.1.Chemical structure of the synthetic *Striga* germination Stimulants GR24

3.1.1.2.1. Preparation of GR24 stock solution

Stock solution of the stimulant was prepared by dissolving 1mg in 1ml of acetone and subsequently completing to volume (100 ml) with sterile distilled water to obtain the desired concentration (10 ppm).

3.2. Methods

3.2.1. Parasitic weed seed cleaning, surface disinfection and conditioning

Seeds of *S. hermonthica* (sorghum or millet strains), *O. crenata* and *P. ramosa* (0.5-1g each) were poured, each, into a measuring cylinder (1000 ml), filled with tap water to which Tween 20 (0.5-1 ml) was added. The measuring cylinder was occasionally swirled. The seeds were allowed to settle and water containing debris and light seeds were decanted. The heavy seeds, separated from sand, by repeated flotation and decantation, were subsequently transferred to a fine sieve (70 μm) and washed with tap water several times to remove traces of the detergent.

For surface disinfection seeds were, immersed for 3 min in 70% ethanol followed by washing with sterilized distilled water and a subsequent immersion for1min in NaOCl solution (1%). The sodium hypochlorite was drained off, and the seeds were washed, under suction with sterilized distilled water several times, until the yellow color disappeared. The seeds, plotted dry on Whatman No.1 filter papers, were air-dried under a

lamina flow cabinet and subsequently stored at ambient temperature till used.

Conditioning of *Striga,O. crenata* and *P. ramosa* seeds were achieved, pending subsequent bioassay procedure, in Petri-dishes using glass fiber filter papers. Surface disinfected seeds sprinkled on 8 mm glass fiber filter papers (GFA) discs (*ca* 25-30 seeds/disc), placed on glass fiber filter papers in Petri-dishes, were moistened with 5 ml of distilled water. The, Petri-dishes, sealed with parafilm, were wrapped in aluminum foil and incubated in the dark at 30^0 C for 14 days for *S. hermonthica* and at 21 °C ±2, in the dark, for 7 days for *O. crenata* and *P. ramosa* seeds.

3.2.2. Surface disinfection and sterilization of sorghum and millet seeds

Millet and sorghum seeds were surface-sterilized by immersion in 1% sodium hypochlorite obtained by dilution of the respective amount of commercial bleach solution (NaOCl), for 5 min. Subsequently, the seeds were thoroughly washed with sterilized distilled water, air dried in a lamina flow cabinet and stored at ambient temperature, till used.

3.2.3. Euphorbia latex extraction

Latex of *E. aegyptiaca* was collected by cutting freshly harvested stems and pressing the cut ends onto a filter paper (What Man No.1). The filter paper, allowed to dry at room temperature, cut into small pieces was placed in a beaker and ethylacetate (200 ml) was added. The set up was placed on a shaker (200 RPM) for (2h) and subsequently filtrated under suction. The filtrate was evaporated to dryness using a rotary evaporator. The residues were collected using 2 ml ethylacetate and kept at 4°C, in brown vials, till used.

3.2.4. Preparation of crude Euphorbia Extract

E. aegyptiaca powder and fresh samples (100 g each) were extracted each with 300 ml of hexane, butanol, chloroform, ethylacetate and ethanol for

three days at ambient temperature with shaking. The extracts were filtrated through filter paper under suction and the filtrate was concentrated using a rotary evaporator as.

3.2.5 Bioassay

3.2.5.1. Bioassay of crude Euphorbia extract

Aliquots (20 µl) of concentrated *Euphorbia* extracts were applied to glass fiber discs and allowed to stand for 2h in a lamina flow cabinet to ensure evaporation of organic solvents (Hexane, chloroform, ethylacetate and Ethanol). The treated discs were overlaid by discs containing conditioned seeds of the parasites (*S. hermonthica, O.crenata* and *P.ramosa*)seeds. Each pair of discs was moistened with 40 µl distilled water. The seeds were re-incubated in the dark at 20^0 C for *O.crenata* and *P. ramosa* and at 30^0 C for *S. hermonthica*. Seeds similarly conditioned and treated with GR24 (0.1ppm for *Striga* and 10ppm for *O. crenata* and *O. ramosa*) or distilled water were included as positive and negative controls, respectively. Germination was examined 24 h later for *S. hermonthica*, and after 7 days for *O. crenata* and *P. ramosa.*

3.2.5.2. Bioassay of crude Euphorbia latex extract

Aliquots (5, 10, 15, 20, 25, 30µl) of *E. aegyptiaca* latex extract were applied to glass fiber discs and allowed to stand for 2h in a lamina flow cabinet to ensure evaporation of ethylacetate. The treated discs were overlaid by discs containing conditioned seeds of the parasites (*S. hermonthica, O. crenata* and *P. ramosa*)seeds. Each pair of discs was moistened with 40 µl distilled water. Seeds treated with GR24 or distilled water were included as controls (see **3.2.5.1**). The seeds were re-incubated in the dark and examined for germination as in **3.2.5.1**.

3.3. Chromatographic behavior of Euphorbia Extract

The chromatographic behaviour of germination stimulants from Euphorbia was investigated using ethylacetate extract. The extract

evaporated to dryness and the residue was dissolved in 2 ml ethylacetate. The extract was subjected to Thin Layer chromatograph (TLC), Column chromatograph (CC) and High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (LC-MS) analysis.

3.3.1. Thin layer chromatography

Aliquots (*ca* 50 µl) of the concentrated ethyl acetate extracts were spotted onto aluminum plates (3.5×8) coated with silica gel 60 F254.

The plates were developed using hexane / ethyl acetate (7:3 and 1:1). Subsequent to development the plates were examined under UV light (254nm) and subsequently cut into 1cm pieces, placed in Petri-dishes and directly assayed for germination inducing activity using conditioned *S. hermonthica* seeds (sorghum, pearl millet strain), *O. crenata* and *P. ramosa* seeds.

3.3.2. Column chromatography

Glass chromatographic columns (34×2 cm) were packed to 15 cm with silica gel (100-200 mesh) obtained from s.d..fiNE-CHEM liMiTEd. The extract (*ca*-0.2 ml) was loaded into the column. The column was eluted with hexane (20 ml) followed in sequence by hexane: ethylactate mixtures (1:0, 9:1, 7:3, 1:1,3:7, 2:8, 1:9 and 0:1v/v). Fractions (10 ml each) were collected and subsequently assayed for germination inducing activity as above using the double discs. Fractions (Fr) showing the highest germination inducing activity (5 and 6) were evaporated to dryness and the residue of each fraction was dissolved in ethylacetate (1ml) and germination inducing activity was confirmed using the double disc technique as above. Further, the concentrates were subjected to TLC using hexane: ethyl acetate (7:3 and 1:1) as developing solvent. The plates were assayed for germination inducing activity as in 3.2.5.2. The samples, subsequently dried, kept in brown vials, were sent to Japan for determination of structures of active compounds.

3.3.3. High Performance Liquid Chromatography LC-Mass condition

Residues of Fr 5 and 6 were dissolved, each, in 300μL of acetone for germination assay and LC-MS analysis (SLs) with high sensitivity to 5-deoxystrigol, strigol, strigylacetate, orobanchol, orobancholacetate, sorgomol and sorgolactone the LC-MS condition described below.

3.4. Statistical analysis

Germinatin data collected from all experiments were transformed to arcsin and subjected to statistical analysis {Analysis of Variance (ANOVA)}, using GenStat package release 10.3DE (PC/Windows 7), VSN International Ltd., UK statistical package (Rothamsted Experimental Station), and SAS 9.1. Means were separated for significance using Duncan Multiple Range Test (DMRT) and Statistix8 for LSD test. Graphs were drawn using Excel 2007 windows 7.

CHAPTER FOUR

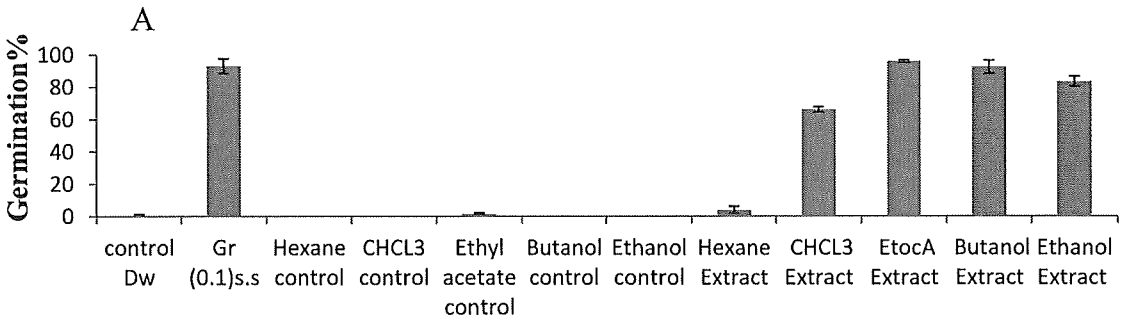
RESULTS AND DESCUSSION

4.1. Bioassay of crude Euphorbia extract

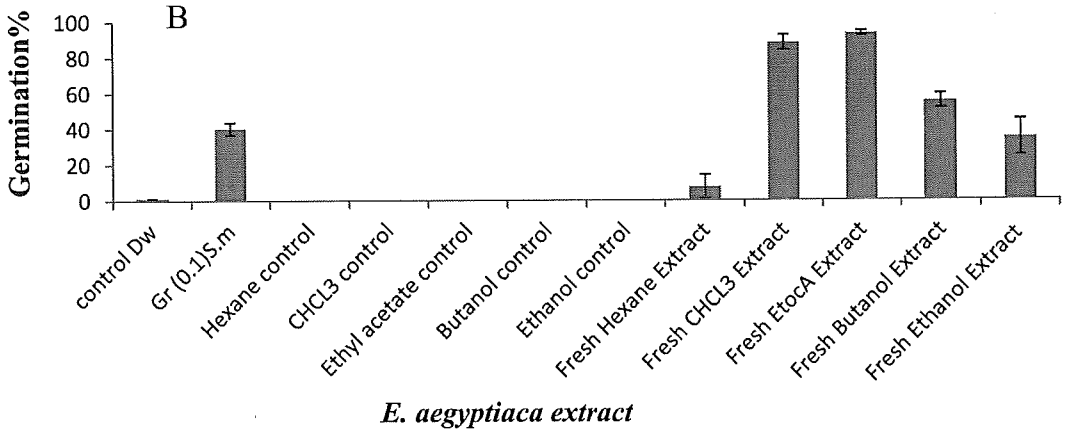
In all experiments four types of parasitic weeds seeds (*S. hermonthica sorghum, S. hermonthica pearl millet, P. ramosa, and O. crenata*) were used to study germination inducing activity and chromatographic behaviour of extracts from *E. aegyptiaca*.

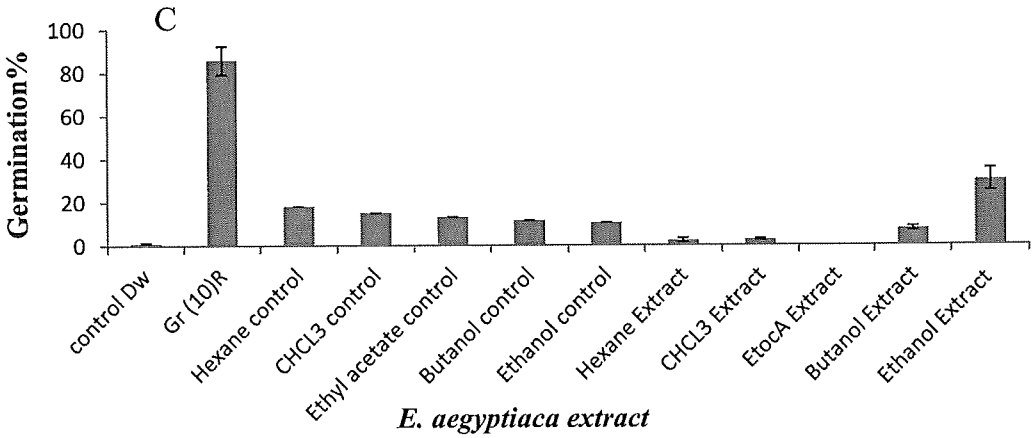
4.1.1. Effects of Fresh *E. aegyptiaca* shoots extracts on germination

The aqueous control treatments induced negligible (0-1%) germination in all parasitic weeds. *S. hermonthica* seeds sorghum and pearl millet strains treated with GR24 displayed high 93 and 40% germination, respectively (Fig 4.1 A and B). *P. ramosa* and *O. crenata* on the other hand, showed 86 and 59 % germination, respectively (Fig 4.1 C and D). Hexane, butanol, chloroform, ethyl acetate and ethanol extracts induced 4, 92, 66, 96 and 83% germination, respectively, in *S. hermonthica* sorghum strains, whereas the corresponding germination figures for its pearl millet congener were7, 55, 88, 93 and 35% respectively. Hexane, butanol, chloroform, ethyl acetate and ethanol extracts induced 6, 8, 3, 0 and 30 % germination, respectively in *P. ramosa*. However, all extracts treatments induced no germination in *O. crenata*, irrespective of the solvent used (Fig 4.1 C and D).



E. aegyptiaca extract





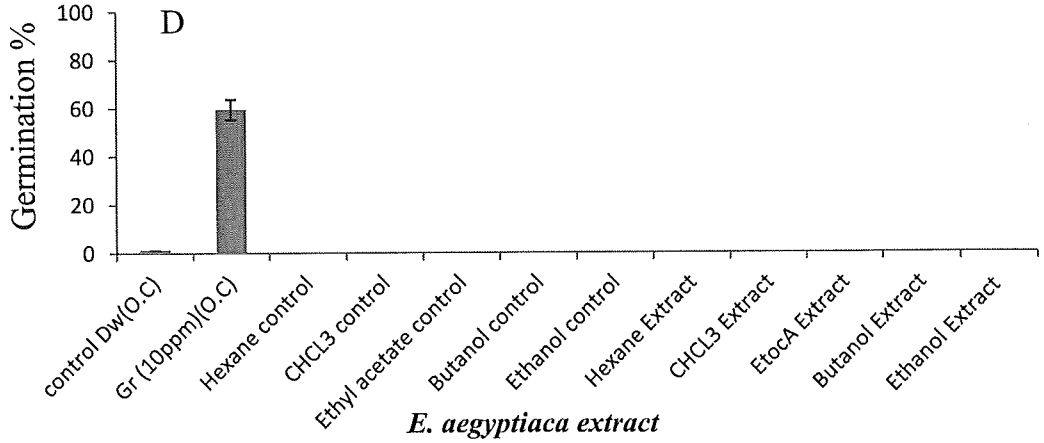


Fig.4.1: Germination inducing activity of *E. aegyptiaca* extracts as influenced by polarity of extracting solvent. A) *S. hermonthica* sorghum strain, B) *S.*

***hermonthica* pearl millet strain, C) *P. ramosa* and D) *O. crenata*. Vertical bars indicate standard error (Fresh *E. aegyptiaca*.**

The results showed that the germination inducing activity varied with species and polarity of the solvent used for extraction of *E. aegyptica*. *S. hermonthica* sorghum strain was more responsive than the its pearl millet congener.

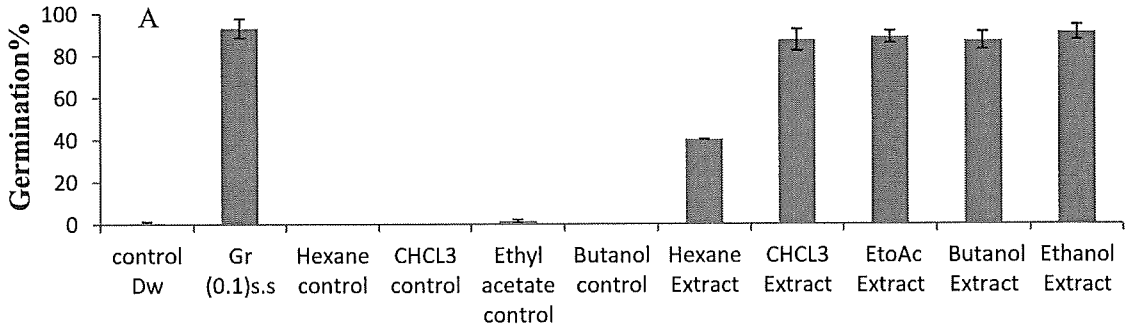
It deserves mentioning that the *S. hermonthica* sorghum strain, showed its lowest germination response to the hexane extract followed by chloroform extract. Further, the parasite seeds showed about equal germination (96, 92, 83 %) in response to the ethyl acetate, butanol and ethanol extracts Fig 4.1 A. *S. hermonthica*, pearl millet strain on the other hand, showed lowest germination in response to hexane, followed by ethanol (35% germination) and butanol (55 % germination) extracts. Germination in response to ethyl acetate and chloroform was the highest and was about equal 93 and 88%) Fig 4.1 B. Such observations showed inconsistency with polarity of solvents and differential response to the stimulants between the two Striga strains. However, the results are consistent with previous reports (Yoneyama *et al.*, 2013; Rezig, 2016) and may be attributed to the nature and/or stereochemistry of the stimulants. *S. hermonthica* sorghum strain is reported to be more, responsive to the strigol type SLs than their *Orobanchol* congeners, while the reverse is true for the pearl millet strain (Rezig, 2016). Further, the results suggest production of a mixture of germination stimulants. Production of mixtures of germination stimulant is in line with the findings of Yoneyama et al., 2010 where several germination stimulants were identified in various plants including hosts and non-host species. *P. ramosa* was by far less responsive to the extracts and the highest response was obtained with the ethanol extract, while *O. crenata* showed no germination, irrespective of the solvent used (Fig. 4.1 C and D). These differences, the existence of which has to be ascertained using root exudates, may have far reaching

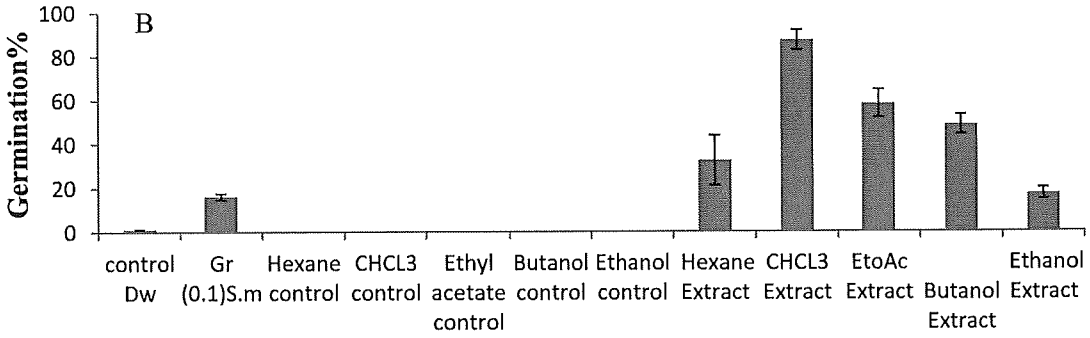
effects on the concept of trap cropping as a means of combating parasitic Orobanchaceae. *E. aegptiaca* is widely distributed in the Sudan (*Abdallah et al.,* 2012).

4.1.2. Effect of dry *E. aegyptiaca* shoots extracts on germination

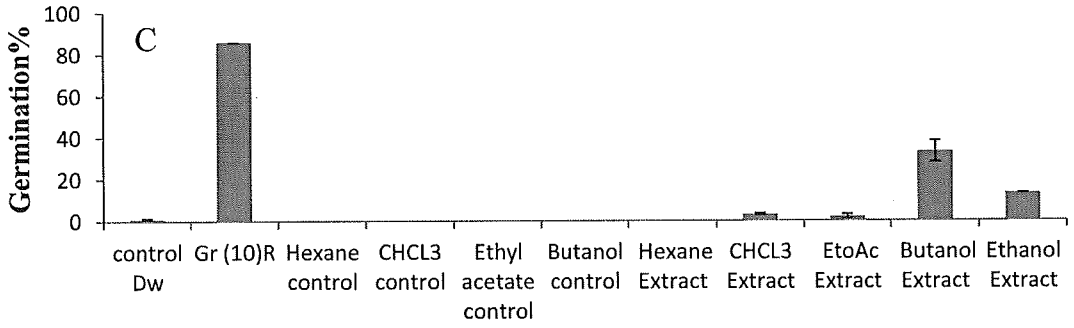
The aqueous control treatments induced negligible (0-1%) germination in all parasitic weeds. *S. hermonthica* seeds sorghum and millet strains treated with GR24 displayed high 93 and 16% germination, respectively (Fig 4.2 A and B). *P. ramosa* and *O. crenata* on the other hand, showed 86 and 59 % germination, respectively (Fig 4.2 C and D).

Induced activity of dry Euphorbia extracts on germination of *S. hermonthica* sorghum strain seeds in response to Hexane, butanol, chloroform, ethyl acetate and ethanol extracts displayed 44%, 87%, 86%, 93% and 88% respectively (Fig 4.2 A).germination of *S. hermonthica* pearl millet strain of Hexane, butanol, chloroform, ethyl acetate and ethanol extracts induced 32%, 49%, 87%, 58% and 17% respectively (Fig 4.2 B). Effect of dry *E. aegyptiaca* extract of *P.ramosa* displayed 0, 33, 5, 5 and 13% germination in response to hexane, chloroform, butanol, ethanol and ethylacetate extracts, respectively (Fig.4.2. C).

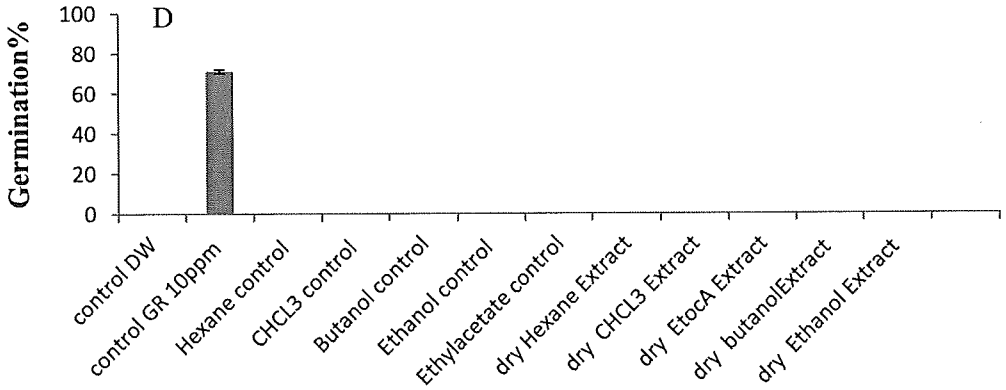




E. aegyptiaca extract



E. aegyptiaca extract

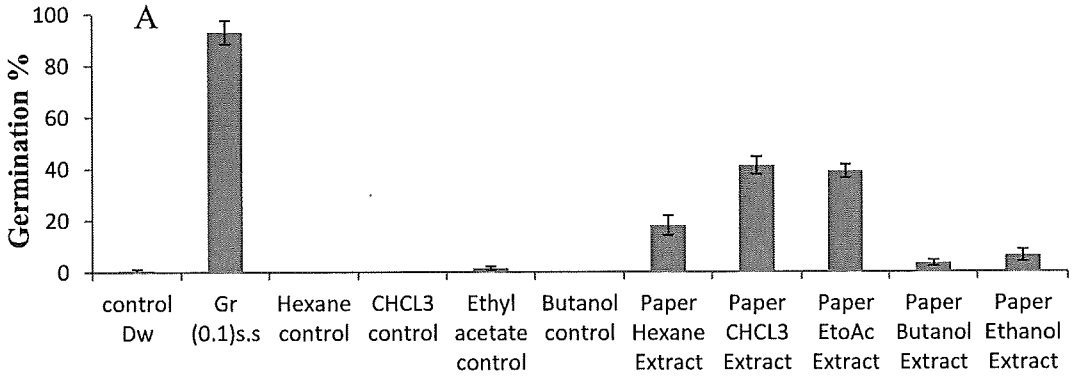


E. aegyptiaca extract

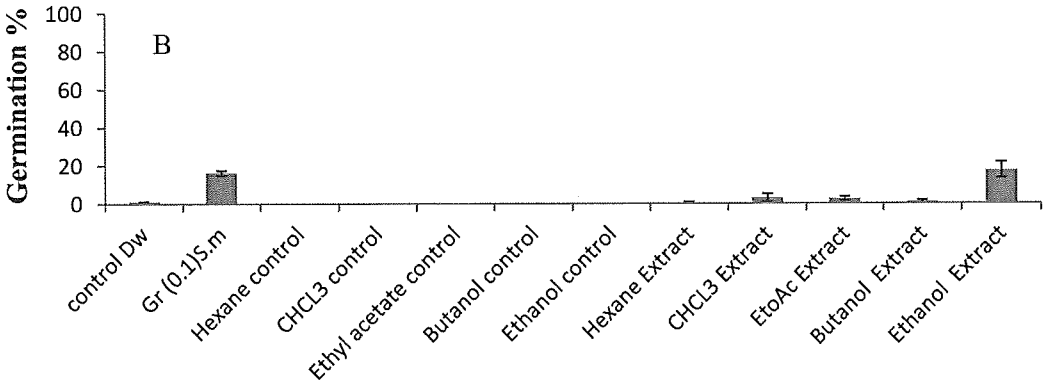
Fig.4.2: Germination inducing activity of *E. aegyptiaca* extract as influenced by polarity of extracting solvent. A) *S. hermonthica* sorghum strain, B) *S. hermonthica* pearl millet strain, C) *P. ramosa* and D) *O. crenata*. Vertical bars indicate standard error.

4.1.3. Effect of Latex Euphorbia extracts on germination

The aqueous control treatments displayed negligible (0-1%) germination in all parasitic weeds. *S. hermonthica* seeds sorghum and millet strains treated with GR24 displayed high 93 and 16% germination, respectively (Fig 4.3 A and B). *P. ramosa* and *O. crenata* on the other hand, showed 86 and 59 % germination, respectively (Fig 4.3 C and D). Hexane, butanol, chloroform, ethyl acetate and ethanol extracts induced 18, 7, 41, 39 and 5% germination, respectively, in *S. hermonthica*, whereas the corresponding germination figures for its pearl millet congener were2, 2, 5, 5 and 17% respectively. Hexane, butanol, chloroform, ethyl acetate and ethanol extracts induced 0, 33, 27, 32 and 10% germination, respectively in *P. ramosa*. However, all extracts treatments induced no germination in *O. crenata*, irrespective of the solvent used.



***E. aegyptiaca* extract**



***E. aegyptiaca* extract**

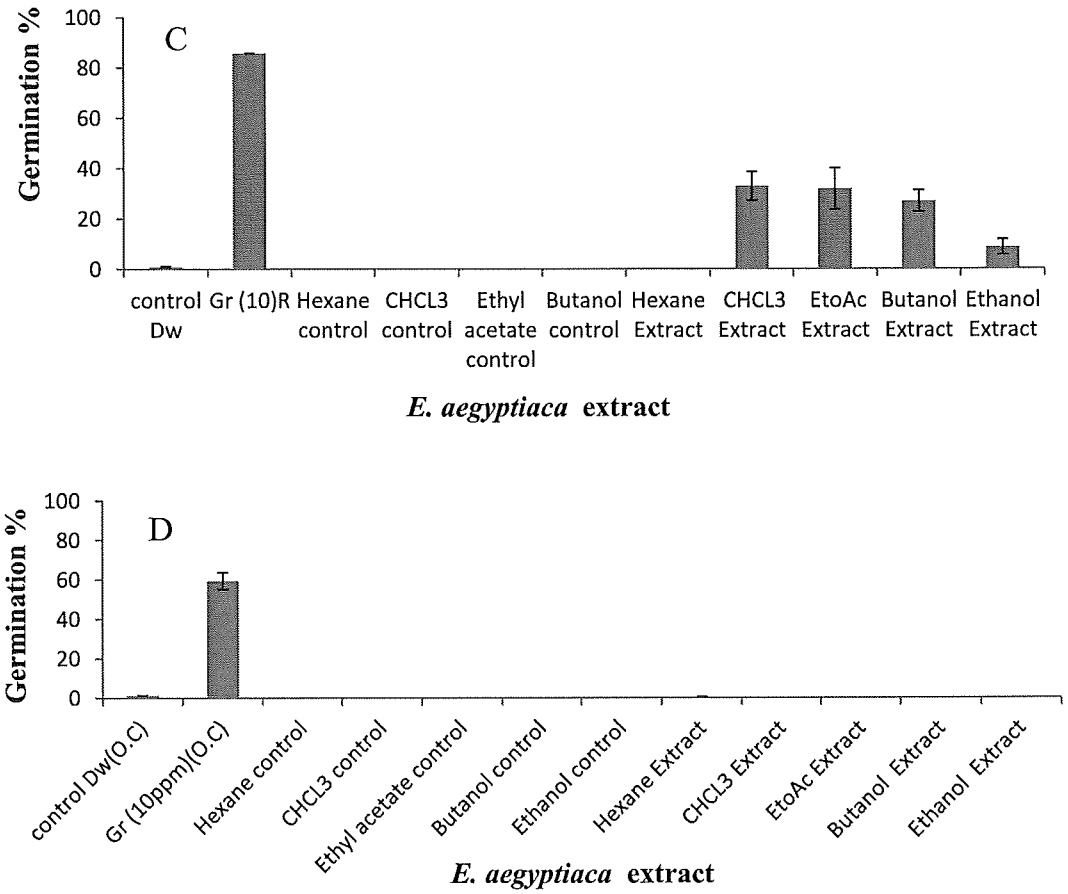


Fig.4.3: Germination inducing activity of *E. aegyptiaca* extract as influenced by polarity of extracting solvent. A) *S. hermonthica* sorghum strain, B) *S. hermonthica* pearl millet strain, C) *P. ramosa* and D) *O. crenata*. Vertical bars indicate standard error.

GR24 induced 74.1, 42.6, 71.2 and 54.2 % germination of *S. hermonthica* sorghum strain, *S. hermonthica*, millet strain, *P. ramosa* and *O. crenata* seeds, respectively. Ethyl acetate extract of Euphorbia latex at 5-30 µl induced 86.7 and 93.7% germination (Table 4.1). Germination progressively increased with concentration, reached a maximum at 20 µl and declined significantly at 25 and 30 µl (Table 4.1). *S. hermonthica* millet strain displayed 45.8% germination. Germination progressively increased, reached a maximum (88.3%) at 25 µl and subsequently declined (Table 4.1). *P. ramosa* on the other hand displayed moderate

germination (64.9-74.6%), which displayed no significant change with changing extract concentration (Table 4.1). *O. crenata* on the other hand displayed no germination.

Table.4.1. Effects of Euphorbia Latex Extract on germination of *S. hermonthica* (pearl millet and sorghum strains), *O. crenata* and *P. ramosa.*

Treatment/ µl	Germination (%)			
	Euphorbia Latex Extraction			
	*S*S	*S*PM	*PR*	*OC*
Control	92.2(74.1)^{AB}	45.8 (42.6)^{E}	89.4 (71.2)^{A}	86.0(54.2)^{A}
5	86.7 (68.6)^{CD}	50.4(45.3)^{E}	74.6(59.7)^{B}	0.00 (0.0)^{B}
10	89.4 (71.0)^{BC}	64.7 (53.5)^{D}	73.7 (59.5)^{B}	0.00 (0.0)^{B}
15	90.8 (72.9)^{AB}	70.5 (57.1)^{C}	64.9 (53.8)^{B}	0.00 (0.0)^{B}
20	93.8 (75.6)^{A}	72.0 (58.1)^{BC}	71.6 (57.9)^{B}	0.00 (0.0)^{B}
25	82.6 (65.4)^{D}	88.3(70.0)^{A}	72.6 (58.5)^{B}	0.00 (0.0)^{B}
30	76.6 (61.1)^{E}	76.4(61.0)^{B}	68.89 (56.1)^{B}	0.00 (0.0)^{B}

2-way ANOVA

Striga **2133.4*****

Stimulant **53.2*****

Striga* Stimulant **57.9*****

Means within a row or a column followed by the same letter(s) are not significantly different (LSD 5%). Data in parenthesis arcsin transformed.

SS=*Striga sorghum*, SPM= *Striga pearl millet*, *PR= Phelipanche ramosa*, OC= *Orobanche crenata.*

In the present work a holistic approach was adopted with the objectives of providing information on activity of *E. aegyptiaca* extracts, root exudates of host plants of *Striga* spp. All extracts from *E. aegyptiaca* (Fresh, dry shoot and latex) samples tested contained a substance or a group of substances, which induced germination of *S. hermonthica* seeds. The

ability of stimulatory substance (s) to induce germination of the two *Striga* strains which are known to be host specific and require different stimulants for germination (Parker and Reid, 1979 and Parker, 1983) suggests involvement of a compound or compounds that have a wide spectrum of activity.

By virtue of this broad-spectrum activity, response to Euphorbia extract, when taken in conjunction with the response to other stimulants, may help in identification of *Striga* species, strains, and physiological variations The occurrence of active substance (s) in *E. aegyptiaca* specie tested, the wide geographical spread of the genus, the broad - spectrum activity of extract and the ease with which it can be obtained might offer cheap and effective means for *Striga* control.

4.2. Chromatographic behavior of Euphorbia Extract:

4.2.1. Thin layer chromatography for separation and purification of the germination stimulant(s) from Euphorbia (Latex):

The mobile phase in all chromatographic experiments was hexane: ethylacetate in different ratios. On thin layer chromatography *E. aegyptiaca* extract displayed high germination inducing activity on *S. hermonthica* sorghum strain (83 and 76%) at Rf 0.14 and 0.29, respectively. it is not possible to distinguish between qualitative and quantitative differences based on bioassay Chromatographic analyses using TLC (Figs. 4.4) did not show clear cut differences in composition of Euphorbia active substances in extract. Mobile phase (7:3) hexane: ethylacetate (Fig.4.4 A and B and C). *S.hermonthica* sorghum strain (83 and 76%) at Rf 0.14 and 0.29 respectively. Very low germination 2, 2 and 1% was displayed at Rf 0.43, 0.71 and 1 respectively. Fig.4.4 (A). *S.hermonthica,* millet strain, showed 32.9 and 4% germination at Rf values of 0.14 and 0.29, respectively (Fig. 4.4 (B). *P. ramosa* on the other hand showed 29, 62 and 15% germination at Rf 0.14, 029 and 0.43,

respectively Fig. 4.4 (C). *O. crenata* on the other hand showed no germination.

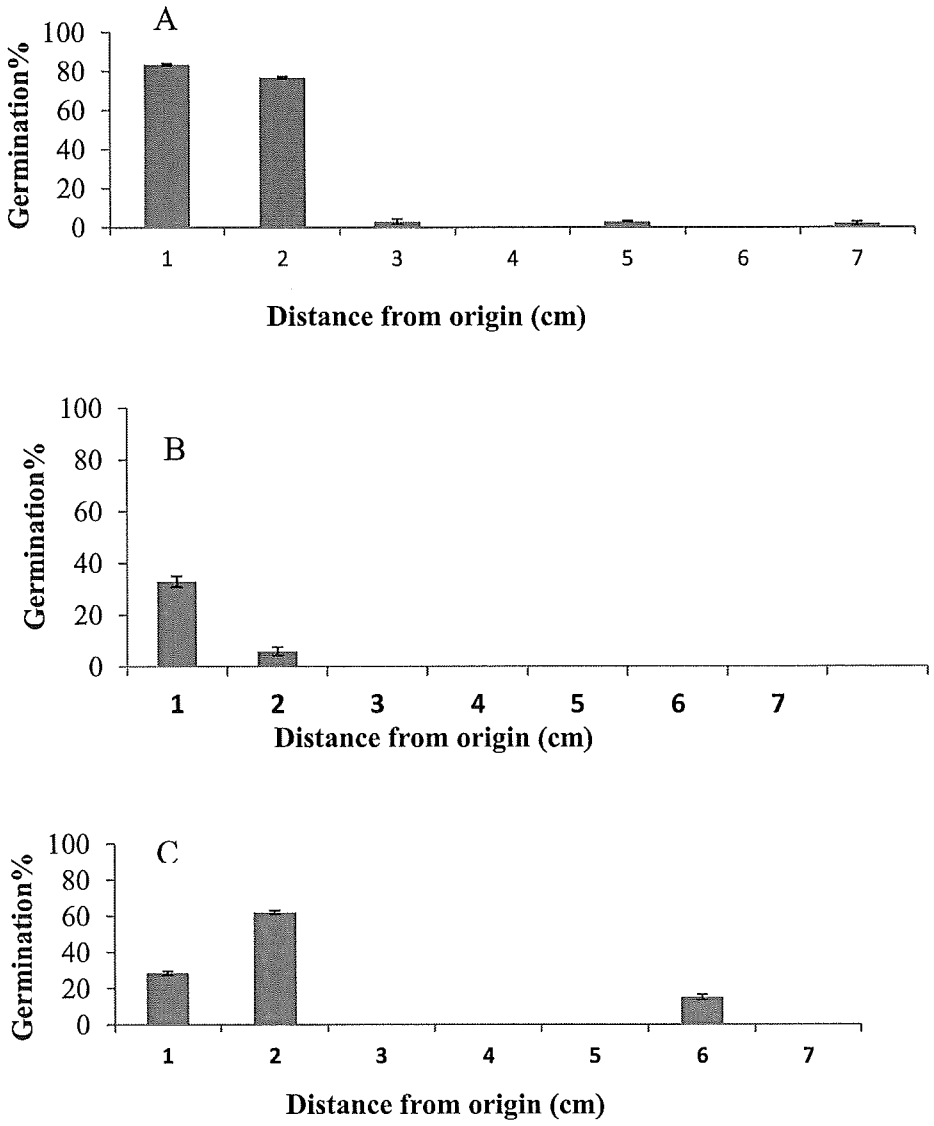
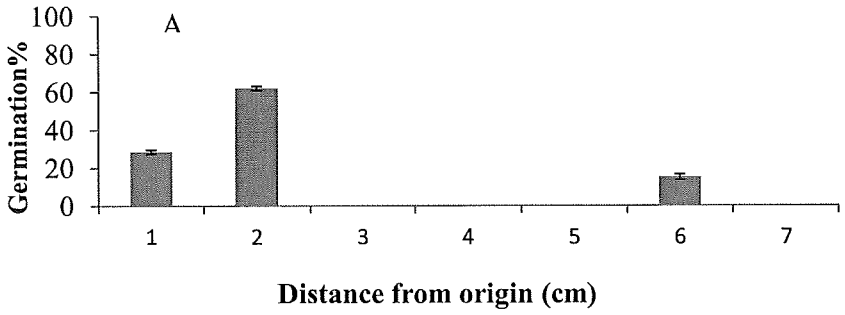


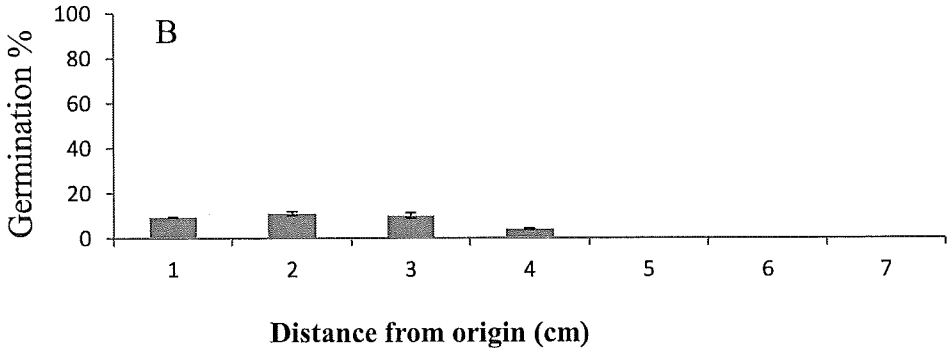
Fig.4.4. Chromatographic behavior of crude *E. aegyptiaca* latex on TLC. A) *S.hermonthica* sorghum strain, B) *S.hermonthica* millet strain, C) *P. ramosa*. Mobile phase (Hexane: ethylacetate (7:3). Vertical bars indicate standard error.

On increasing polarity of mobile phase (Hexane: ethylacetate 1:1) *S. hermonthica,* sorghum strain, showed 29, 62 and 15% germination at Rf values of 0.14, 0.29 and 0.86 respectively Fig. 4.5 (A). *S. hermonthica* millet strain, on the other hand displayed 9, 11, 10 and 4% at Rf values of

0.14, 0.29, 0.43, 0. 0.71. Respectively Fig. 4.5 (B). *P. ramosa* on the other hand displayed 20, 4, 33 and 49 % at Rf values of 0.14, 0.43, 0.71 and 0.86, respectively (Fig. 4.5 (C). *O. crenata* showed no germination response.

The differential germination response shown by the different parasites species indicates differential sensitivity of the seeds to germination stimulants from *E. aegyptiaca*. Differences in response between *S. hermonthica* sorghum and millet strains has been reported by several authors (Kim *et al.*, 1994). *P. ramosa* appears to be less sensitive to germination stimulants from *E. aegyptiaca. O. crenata*, on the other hand is by far less responsive as it showed no germination response.





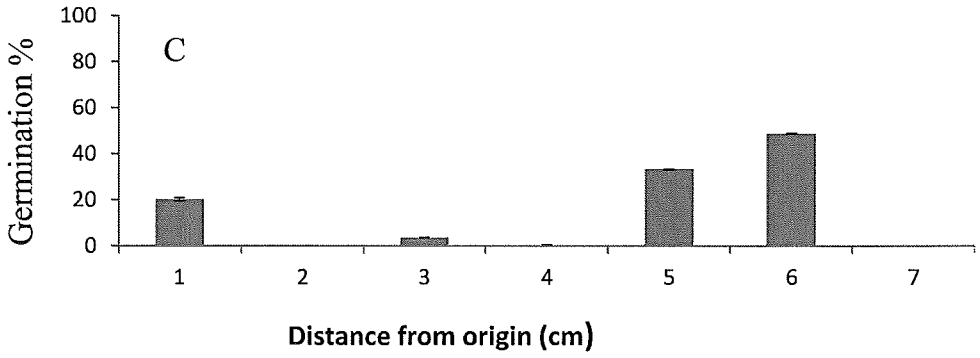


Fig. 4.5. Chromatographic behavior of crude E.aegyptica latex on TLC. A) *S.hermonthica* sorghum strain, B) *S.hermonthica* millet strain, C) *P. ramosa*. Mobile phase (Hexane: ethylacetate (1:1). Vertical bars indicate standard error.

4.2.2. Column chromatography

4.2.2.1. Column chromatography of crude Euphorbia latex

Hexane eluate of crude Euphorbia latex showed negligible (4%) germination inducing activity on *S.hermonthica* (sorghum strain) (Fig. 4.6. (A). Increasing proportion of ethylacetate in the solvent mix from 10 to 50% showed negligible to little increase in germination inducing activity (4-12.5%).) Fig.4.6 (A). However, a surge in germination inducing activity (80% germination) was observed on increasing the proportion of ethylacetate to 70%. A further increase in ethyl acetate to 90 and 100% resulted in reduced germination.

S. hermonthica millet strain showed negligible germination (0-1%) when the ethyl acetate proportion in the developing solvent was 50% or less (Fig. 4.6. B). Increasing the ethyl acetate proportion in the developing solvent to 70% resulted in maximum germination (29%) (Fig 4.6. B). A further increase in ethyl acetate proportion to 90 and 100% reduced germination to 14and 4%, respectively. For *P. ramosa* fractions with low polarity (ethylacetate <70%) induced no germination. Increasing the ethyl acetate proportion in the developing solvent to 70, 90 and 100% resulted in 29, 14 and 3% germination respectively. (Figs. 4.6. (C). *O. crenata*

showed no germination response, irrespective of the ethyl acetate proportion in the developing solvent.

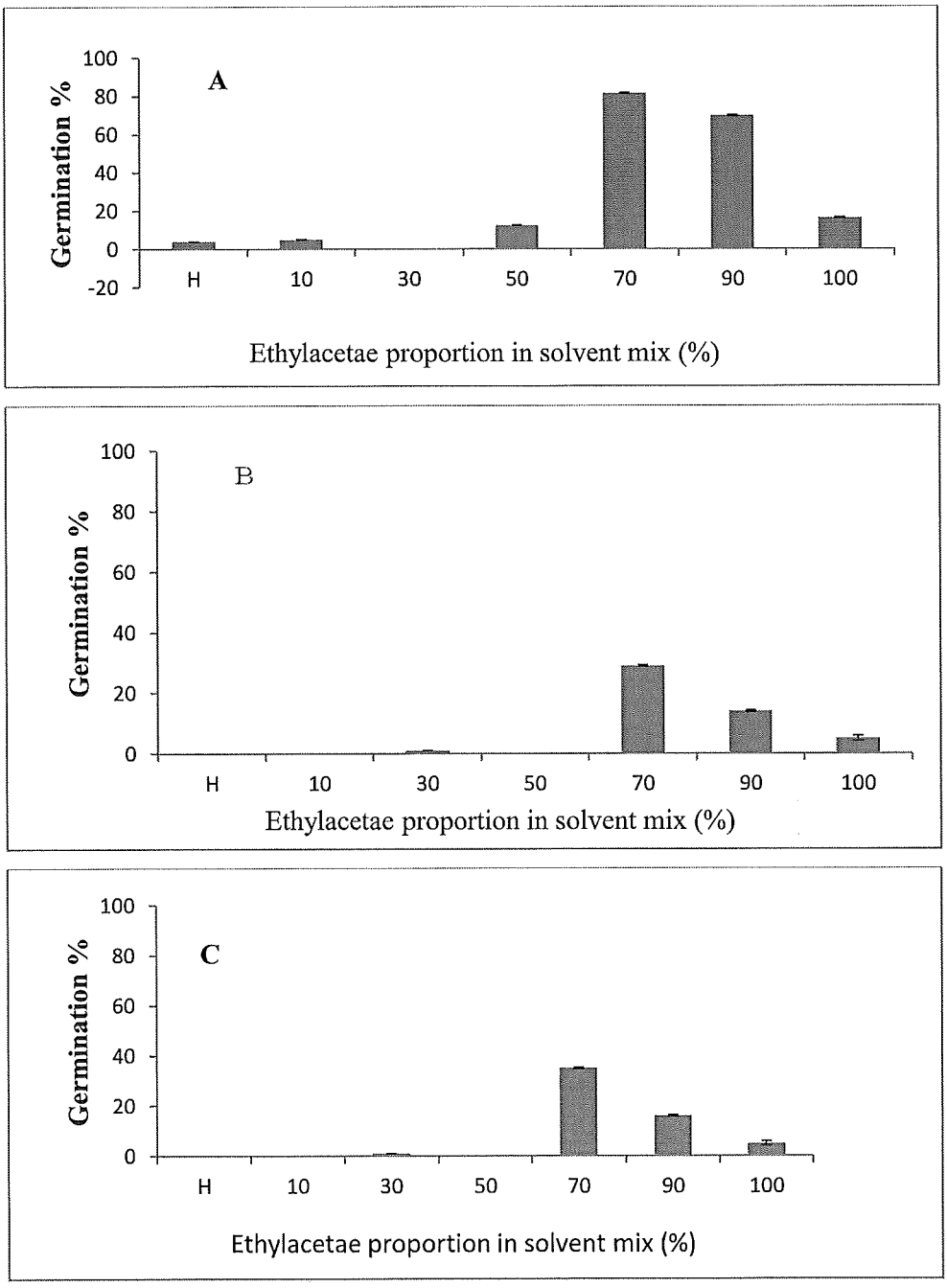
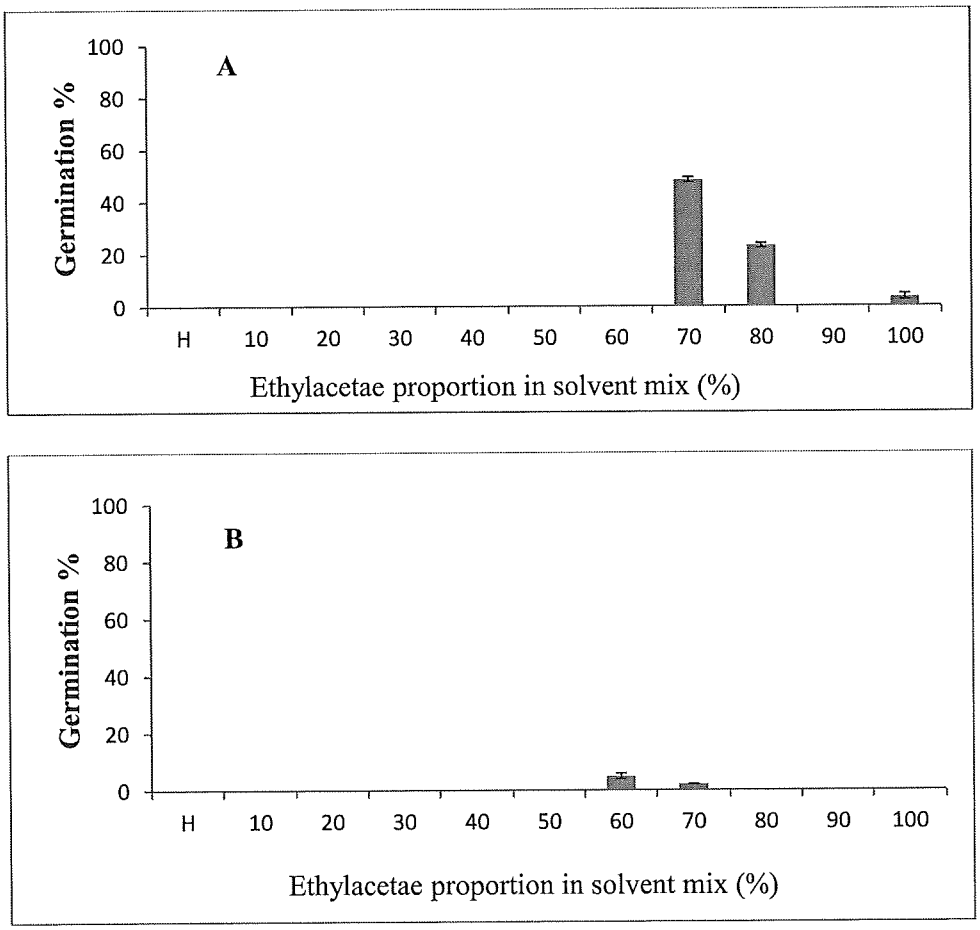


Fig. 4.6. Chromatographic profile of germination inducing activity of *E.aegyptica.* latex extract. A) *S. hermonthica* sorghum strain, B) *S. hermonthica* millet strain, C) *P. ramosa* (Column chromatography). H =hexane. Vertical bars indicate standard error.

4.2.2.2 Column chromatography of dry Euphorbia sample

Colum chromatography of dried *E. aegyptiaca* shoots showed a different profile when compared with the extracts of Euphorbia latex (Fig. 4.7 A-C). Hexane and hexane/ ethyl acetate mixtures where the proportion of the ethyl acetate was less than 60% induced no germination in all parasite seeds. Increasing the ethyl acetate proportion in the developing solvent to 70, 80, 90 and 100% induced 48, 23, 0 and 3% germination in *S. hermonthica* sorghum strain (Fig. 4.7 A). The corresponding germination figures for the pearl millet strain were 5, 3, 0 and 0 %, respectively. *P. ramosa*, on the other hand, showed high germination (77%) only when the ethyl acetate proportion in the developing solvent was increased to100% Fig 4.7 (C). *O. crenata* showed no germination, irrespective of the composition of the developing solvent.



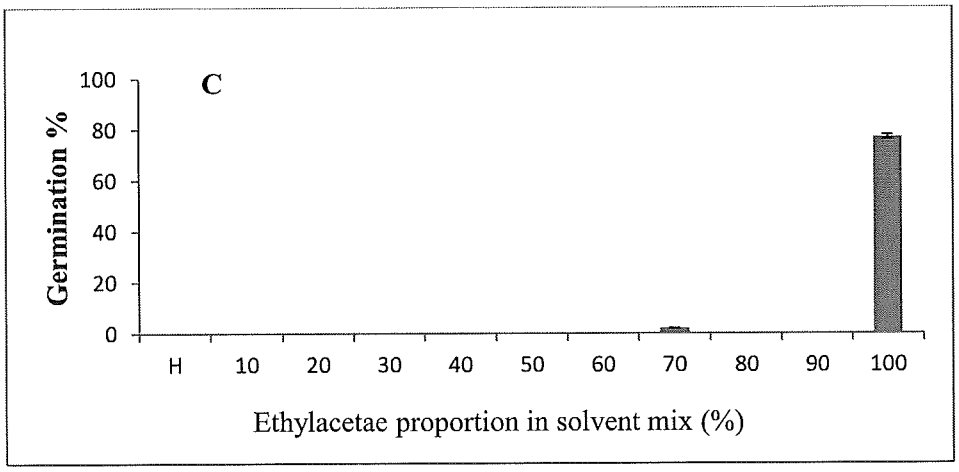


Fig. 4.7. Chromatographic profile of germination inducing activity of *E.aegyptica.* latex extract. A) *S. hermonthica* sorghum strain, B) *S. hermonthica* millet strain, C) *P. ramosa* (Column chromatography). H =hexane. Vertical bars indicate standard error.

4.2.3. High Performance Liquid Chromatography LC-Mass analysis for *E.aegyptica.* Fractions (5 and 6)

The LC-Mass analysis showed no chromatogram comparable to that of the known strigolactones (5-deoxystrigol, strigol, strigylacetate, orobanchol, orobanchulacetate, sorgomol and sorgolactone) (Fig 4.9 A and B).

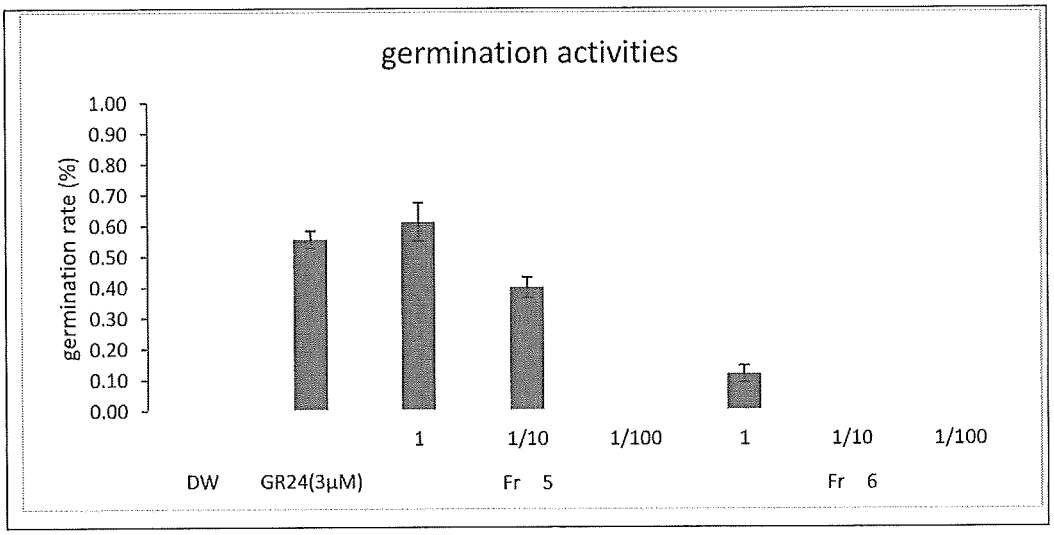


Fig.4.8. Chromatographic behavior of active Euphorbia latex fractions from Column chromatography. Vertical bars indicate standard error.

The undiluted and 10 fold diluted fractions induced 60% and 40% germination respectively (Fig 4.8)

Failure to detect a strigolactone in the active fractions of *E. aegyptiaca* latex suggesting that the active ingredient(s) in the fraction may be a novel SL or a non-SL compound. However, the possibility that the level of strigolactone in the extract was below the deduction limit cannot be ruled out. Germination assays are reported to be at least 100-fold more sensitive than mass spectrometry for the detection of SLs (Yoneyama *et al.*, (2010).

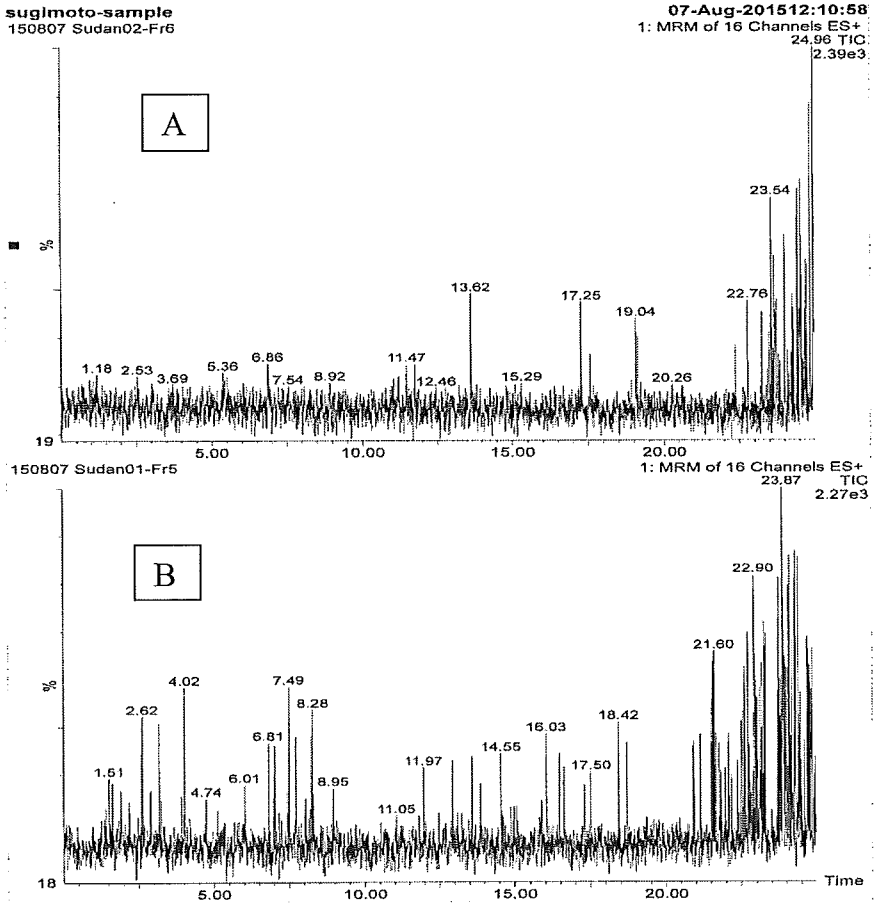


Fig.4.9. Chromatogram of *S. hermonthica* germination stimulants from *E. aegyptiaca*. A) Fractions 6 and B) fraction 5.

Conclusions

- The ability of stimulatory substances in Euphorbia to induce germination of a range of parasitic Orobanchaceaeincluding *S. hermonthica*, sorghum and millet strains, and *P. ramosa*, but not *O. crenata* indicated production of a mixture of compounds or a compound of a wide spectrum of activity.
- The stimulant(s) produced by *E. ageytriga* are polar. However, germination of the different species did not always show a single pattern which could be synchronized with the polarity of the solvent used for extraction. Thus suggesting species specific inhibitors and/or promoters of germination with different polarities.

Recommendation

- *E. aegyptiaca* is widely distributed in Sudan. *Striga* and *P. ramosa* germination as well as mycorrhizal colonization of plant roots are stimulated by Strigolactones. The possibility of reducing parasitism directly via depletion of *Striga* and *P. ramosa* seed bank, directly through suicidal germination or indirectly through mycorrhization has to be investigated.
- Further work leading to identification of the active germination stimulants in *E. aegyptiaca* extract is imperative as it may lead to synthesis of novel more stable analogues with similar activity.

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Appendix I

Appendix 1: Some of the most important parasitic weeds according to the economical losses they cause.

Family	Genus	species	Host crop
Orobanchaceae	*Striga*	*S. hermonthica*	Sorghum, maize, millet
		S.asiatica	Maize, Sorghum
		S.gesnerioides	Cowpea
	Orobanche	*O.cernua (O.cumana)*	Sunflower, tomato, Tobacco
		O.crenata	green pea, lentils, broadbean,
		O.ramosa/ O. aegyptiaca	chickpea, carrot, celery
			Onion, lettuce, Sunflower, Broadbean
		O. minor	green pea, lentils
			chickpea, tomato,
	Agallinis alectra	A. purpurea	Tobacco, potato,
		A.vogelii	carrot, celery,canola
			Lettuce, broadbean
			Tobacco,carrot,
			celery, red clover.
			Cowpea

Appendix II

Appendix II: Thin layer chromatography results of selected parastic weeds.

Parasitic weed type	Mobile phase Hexane: Ethyl acetate	Rf value	Germination%
S.hermonthica Sorghum strain	7:3	0.14	83
		0.29	76
		0.43	2
		0.71	2
		1	1
S.hermonthica millet strain	7:3	0.14	33
		0.29	4
P.ramosa	7:3	0.14	29
		0.29	62
		0.43	15
S.hermonthica Sorghum strain	1:1	0.14	29
		0.29	62
		0.86	15
S.hermonthica millet strain	1:1	0.14	9
		0.29	11
		0.43	10
		0.71	4
P.ramosa	1:1	0.14	20
		0.43	4
		0.71	33
		0.86	49

Appendix III

LC-MS condition

Column 100*2.0 mm, 2.5 um, COSMOSIL Packed column 2.5C18-MS-II, 30° C, solvent system is MeOH/H_2O (1:1 to 7:3) and Flow rate is 0.2 ml/min.

MS-condition Positive electro spray ionization mode: Capillary voltage 3 kV, Source temp120 °C, desolvation gas temp 350 °C Nebuliser N_2 gas 50 L/h. desolvation N_2 gas, desolvation N_2 gas550 L/h, Collision-induced dissociation argon at 0.1 ml/min.