

**Sudan University of Science and
Technology
College of Graduate Studies**

**In Vitro Effect
of *Punica granatum* and *Nigella
sativa* Extracts on *Schistosoma
mansoni* Cercariae, Miracidia and
Adult Worms**

**Thesis presented For Fulfillment of
Ph.D. in Parasitology**

by

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**بِسْمِ اللّٰهِ الرَّحْمٰنِ
الرَّحِیْمِ**



فِيهِمَا فَاكِهَةٌ وَبَخْلٌ وَرُمَّانٌ " (الرحمن: 68)
 وَهُوَ الَّذِي أَنشَأَ جَنَّاتٍ مَّعْرُوسَاتٍ وَعَيْبَرٌ مَّعْرُوسَاتٍ وَالنَّخْلَ وَالزَّرْعَ مُخْتَلِفًا
 أَكْلُهُ وَالزَّيْتُونَ وَالرَّمَّانَ مُتَشَابِهًا وَعَيْبَرَ مُتَشَابِهًا كُلُوا مِن ثَمَرِهِ إِذَا أَثْمَرَ وَآتُوا
 حَقَّهُ يَوْمَ حَصَادِهِ وَلَا تُسْرِفُوا إِنَّهُ لَا يُحِبُّ الْمُسْرِفِينَ " (الأنعام: 141).
 وَجَنَّاتٍ مِّنْ أَعْنَابٍ وَالزَّيْتُونَ وَالرَّمَّانَ مُشْتَبِهًا وَعَيْبَرَ مُتَشَابِهًا انظُرُوا إِلَى
 ثَمَرِهِ إِذَا أَثْمَرَ وَيَنْعِهِ إِنَّ فِي ذَلِكُمْ لَآيَاتٍ لِّقَوْمٍ يُؤْمِنُونَ " (الأنعام: 99).



قال الله تعالى :

" وما ينطق عن الهوى إن هو إلا وحي يوحى " (النجم: 2-4)

يقول صلى الله عليه وسلم: "عليكم بهذه الحبة السوداء.. فإن فيها شفاء لكل داء إلا السام " رواه البخاري "

Dedication

In the memory of my parents

with affection and gratitude,

To My Wife and Children

for a best future

In memory of my best friend,
Mr. Bayoumi El-Senoussy

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ABSTRACT

ABSTRACT

Schistosomiasis, is a disease caused by a blood borne flukes. It is the second most prevalent tropical disease after malaria and it causes great public health and socio-economic problems in developing countries. More than 200 million people suffer from the disease and about 500-600 million people in 74 countries are at risk of infection.

Synthetic compounds have been used to interrupt the life cycle of *Schistosoma*. However, right concentrations, these compounds are harmful to the environment. Also some other synthetic compounds, the most popular of which is praziquantel are used in treatment of infection with bilharzia; however, there are some reports of tolerance of the worm to higher concentrations of the drug. Therefore, many medicinal plants have been screened to disrupt one or more stage of *Schistosoma* life cycle, as they are safer to the environment than synthetic compounds. In this study, tannins and alkaloids of different parts of pomegranate as well as saponins and alkaloids of black seeds were tested for their biocidal activity against miracidia, cercariae and adult worms. All tested tannins and alkaloids were lethal to miracidia, cercariae and adult worms.

Miracidia were the most sensitive to the lethal effect of the tested compounds. A concentration as low as 0.39 ppm of tannins was enough to kill 100% of miracidia after 50-150 min and to kill 50% of miracidia within 25-50 min. At a higher concentration (50 ppm) the lethal time for 100% of miracidia ranged between 5 and 15 min and the lethal time for 50% mortality of miracidia ranged between 0.5 and 6 min. Placenta tannins were the most potent tannins of the tested pomegranate tannins.

Fifty ppm pomegranate root and stem alkaloids killed 100% miracidia after 0.6 and 50 min respectively and 0.39 ppm killed 50% miracidia after 0.6 and 21.5 min respectively.

Nigella sativa alkaloids and saponins were lethal to 100% miracidia at 50 ppm after 5 and 25 min respectively, while 50% miracidia

were killed after 0.6 and 16.6 min respectively. At a low concentration of 0.39 ppm, 100% of miracidia were killed after 150 and 180 min respectively and 50% miracidia were killed after 41 and 51 min.

Amongst the 6 tested compounds purified from pomegranate stem alkaloids were the most potent cercaricides, followed by, placenta tannins, rind tannins, root tannins, stem tannins and root alkaloids respectively. The $LT_{100\%}$ was achieved in a time range of 50 to 700 min at 0.39 ppm and 5 to 60 min at 50 ppm. On the other hand, the $LT_{50\%}$ was achieved after 47 to 300 min at 0.39 ppm and 0.7 to 40 min at 50 ppm.

Stem alkaloids which were the most potent tested compounds of pomegranate killed 50% of cercariae after 0.7 and 37 min at concentrations levels of 50 and 0.39 ppm and killed 100% cercariae after 5 and 50 min at concentrations of 50 and 0.39, respectively.

Black seeds extract killed 100% of the tested cercariae after 35-330 min in case of alkaloids and 50-400 min when saponins were used in concentrations ranging between 50-0.39 ppm were tested. On the other hand, the $LT_{50\%}$ was achieved after 19.3-127.1 min when alkaloids were used and 27.8-312.1 min when saponins were used at concentrations ranging between 0.39 and 50 ppm.

The eight compounds extracted from pomegranate and black seeds were tested *in vitro* for their lethal effect on adult worms and were compared with praziquantel at three concentrations (25, 50, and 100 ppm).

All tannins were lethal at 100 ppm to effect 100% mortality in worms after 24-120 hr. The most powerful tannin on *S.mansoni* adult worm was rind tannin. After 120 hours, 40% mortality was observed at 25ppm. At a concentration of 50 ppm, 20, 60 100% of worms were killed after, 72, 96 and 120 hours respectively. At 100 ppm, 20, 60 100% of worms were killed after 24, 48 and 72 hr respectively.

It should be mentioned that worms looked paralyzed and deformed, even at non-lethal low concentrations of pomegranate tannins.

Alkaloids from the root of *P. granatum* caused 50% and 100 % mortality of *S. mansoni* after 24 h and 48 h, respectively at 100 ppm

concentration. None of the tested worms died at the other lower concentrations till the fifth day of the experiment.

N. sativa alkaloids were less potent than pomegranate stem alkaloids. None of the tested concentrations had any effect after the first 72 h of the experiment. After 96 and 120 h at 100ppm, 25% and 50 % mortality of the worms was observed respectively. On the other hand 25 and 50 ppm were lethal to 25 % of the worms after 120h.

Nigella sativa saponins also showed a higher lethal effect than alkaloids on worms. Fifty percent mortality of the worms was observed after 24 h and 100% mortality was observed after 48 h. At 50 and 25 ppm, 25% and 50% of the worms died, respectively after 120 h.

Praziquantel which is the drugs of choice for treatment of Schistosomiasis was used as a positive control. One hundred percent mortality of *S. mansoni* adult worms was observed after 48 h for all the concentrations used (25 ppm, 50 ppm, and 100 ppm). After 24 h 50% of worms were killed at 25 ppm and 75% of worms were killed by both 50 ppm and 100 ppm.

Saponins of black seeds and rind tannins of pomegranate exist abundantly in both plants and they were found to have a potent lethal effect on adult schistosomes. Therefore, they were chosen for further investigation of their effect on the tegument using scanning electron microscope.

Both drugs were found to cause changes in the topography of the worms and the effect of both drugs was dependent on the dose used.

Saponins caused, swelling of the tegument, wrinkling and fusion of tegumental ridges, loss of tubercles, peeling. Peeling caused by saponins was severe enough to produce large ulcers in both male and female worms.

On the other hand, rind tannins of pomegranate caused, erosion of tegument, though not to the extent of ulceration as in the case of *N. sativa* saponins. It also caused wrinkling, swelling, loss of spines and degeneration and rupture of tubercles and this was associated with

blebbing. The detrimental effect of tegumental damage was discussed in the thesis.

Severe damages to suckers of the worms were observed by both rind tannins of pomegranate and saponins of black seeds which might result in a loss of the ability of worms to ingest blood and to attach themselves to blood vessels.

Histopathological study of worms treated with *P. granatum* tannins and *N. sativa* saponins revealed a severe damage of sub-tegumental layers including longitudinal and circular muscle fibers, which suffered from focal lysis, vacuolization, and degenerations.

Total protein profile of adult worms (both males and females) treated with rind tannins and of *P. granatum* and saponins of *N. sativa* was investigated by SDS- polyacrylamide gel electrophoresis. Changes in 6 proteins of different molecular weights were detected. Proteins of molecular weights, 59, 18 and 13 kDa decreased in amounts compared to untreated worms, while a protein of a molecular weight, 55 kDa, increased in amounts. Possible effect of level of protein expression observed energy production, viability and morphology of worms was discussed.

مستخلص الدراسة

تم فحص تأثير تركيزات تخفيفات مختلفة من المركبات المستخلصة من نبات الرمان ونبات حبة البركة على كل من المراسيديا والسركاريا والدودة البالغة في أطباق إليزا وسجلت النتائج بعد فترات زمنية مناسبة.

- و في حالة الدودة تم اخذ بعض الديدان المعالجة بالمركبات النباتية المستخلصة وعمل التالي:
- 1- فحص الديدان بالمجهر الالكتروني الماسح بعد تثبيتها وتجفيفها وطلاتها بالذهب للتعرف على تأثير المركبات النباتية تحت الدراسة على طبقة التجيومنت الخارجية للدودة.
 - 2- ثبتت بعض الديدان وطمرت في الشمع وصبغت لعمل قطاعات لفحصا نسيجيا للتعرف على تأثير المركبات النباتية على التركيب الداخلي للدودة.
 - 3- خلط الديدان وفرمها في محلول لإذابة البروتينات تمهيدا لفصلها كهربائيا على هلام الأكريل و صباغة الهلام وفحص البروتينات المفصلة.

كانت المراسيديا أكثر حساسية للتأثير القاتل للمواد المستخلصة قيد الدراسة حيث قتلت المواد الثمانية المستخلصة المراسيديا بنسبة 100% بعد فترة زمنية قصيرة من 50 إلى 150 د قيقة و قتلتها بنسبة 50% بعد 25 إلى 50 د قيقة عند تركيز 0.39. جزء في المليون أما عند استخدام تركيزا م قدره 50 جزء في المليون فقد قتلت المراسيديا بنسبة 100% خلال من 5 إلى 15 د قيقة و قتلتها بنسبة 50% في وقت صغير بلغ من 0,5 إلى 6 د فائق. هذا ولا قد كانت تانيئات البلاسنتا لثمرة الرمان الأكثر فعالية بين التنيئات المختبرة.

تمكنت ال قلويدات المستخلصة من الجذر والساق عند تركيز 50 جزء في المليون من قتل 100% من المراسيديا بعد فترة زمنية قدرها 0,6 و 50 د قيقة على التوالي. أما عند تركيز 0,39 جزء في المليون من قلويدات الجذر والساق فقد تم قتل 50% من المراسيديا في فترة زمنية قدرها 0,6 و 21,5 د قيقة على التوالي.

من ناحية تمكنت قلويدات وصابونيات الحبة السوداء من قتل 100% من المراسيديا عند تركيز 50 جزء في المليون بعد 5 و 25 د قيقة على التوالي , بينما قتل 50% من المراسيديا تم بعد 0.6 و 16.6 على التوالي. أما عند التركيز 0.39 جزء في المليون فأمكن قتل المراسيديا بواسطة ال قلويدات والصابونيات بنسبة 100% بعد 150 و 180 د قيقة على التوالي و قتلها بنسبة 50% بعد 41 و 51 د قيقة على التوالي.

ضمن الستة مواد المختبرة في هذه الدراسة والمذقاة من نبات الرمان كانت قلويدات ساق الرمان الأكثر كفاءة في قتل السركاريا, تليها تانيئات البلاسنتا و قشرة الثمرة والجذور والساق و قلويدات الجذر على التوالي. و قد تطلب قتل 100% من السركاريا فترة زمنية تراوحت من 50 إلى 700 د قيقة عند تركيز 0.39 جزء في المليون وتراوحت بين 5 إلى 60 د قيقة عند تركيز 50 جزء في المليون. أما قتل 50% من السركاريا فقد تطلب 47 إلى 300 د قيقة عند تركيز 0.39 جزء في المليون و 0.7 إلى 40 د قيقة عند تركيز 50 جزء في المليون.

قلويدات ساق الرمان وهي الأكثر كفاءة في قتل السركاريا ضمن المركبات المدروسة قتلت 50% من السركاريا بعد 0.7 و 37 د قيقة عند تركيز 50 و 0.39 جزء في المليون على التوالي و قتلت 100% من السركاريا بعد 5 و 50 د قيقة عند تركيز 50 و 0.39 جزء في المليون على التوالي.

أما مستخلصات الحبة السوداء فقد قتلت 100% من السركاريا بعد 35 - 330 د قيقة في حالة ال قلويدات و 50 - 400 د قيقة في حالة الصابونيات عند استخدام تركيزات تراوحت من 50 إلى 0.39 جزء في المليون و تم قتل 50% من السركاريا بعد 19.3 - 127.1 د قيقة عند استعمال ال قلويدات و 27.8 - 312.1 د قيقة عند استعمال الصابونيات عند تركيز تراوح بين 0.38 و 50 جزء في المليون.

هذا و قد تم دراسة التأثير القاتل للمركبات الثمانية المستخلصة من نبات الرمان والحبة السوداء على الدودة البالغة و قورنت في كفاءته مع البرزيكوانتل عن تركيزات ثلاث وهي 25 و 50 و 100 جزء في المليون . كل التانيئات كانت قاتلة عند تركيز 100 جزء في المليون 100% من

الديدان بعد 24 - 120 دقيقة. ولقد كانت أقوى المركبات المستخلصة تانينات قشرة ثمرة الرمان فبعد 120 دقيقة، قتلت 40% من الديدان عند تركيز 0.25 جزء في المليون وعند تركيز 50 جزء في المليون قتلت 20, 60, 100% من الديدان بعد 72, 96, 120 ساعة على التوالي. أما عند تركيز 100 جزء في المليون فإن 20, 60, 100 من الديدان قتل بعد 24, 48 و 72 ساعة على التوالي.

و جدير بالذكر أن الديدان كانت أثناء معالجتها بواسطة التانينات تبدو مشلولة فيما عدا حركات بطيئة عند مؤخرتها وقد حدث هذا حتى عند تركيزات أقل من التركيزات الـ قاتلة. فلويدات جذر الرمان سببت مقتل 50% و 100% من الديدان بعد 24 ساعة و 48 ساعة على التوالي عند تركيز 100 جزء في المليون. وفشلت التركيزات الأقل في قتل الديدان حتى اليوم الخامس.

فلويدات الحبة السوداء كانت أقل كفاءة مقارنة بتانينات الرمان. وبالتالي لم يظهر تأثيرها القاتل حتى اليوم الثالث. وعلى كل حال فقد ماتت 25% و 50% من الديدان بعد 96 ساعة و 120 ساعة على التوالي عند تركيز 100 جزء في المليون وماتت 25% من الديدان بعد 120 ساعة عند تركيزي 25 و 50%.

وقد أظهرت صابونيات الحبة السوداء كفاءة أكثر من الفلويديات حيث أنها قتلت 50% من الديدان بعد 24 ساعة و قتلت 100% من الديدان بعد 48 ساعة عند تركيز 100 جزء في المليون. أما عند تركيزي 50 و 25 جزء في المليون فقد قتلت بعد 120 ساعة 25 و 50% من الديدان على التوالي. أما بالنسبة للبرازيكوانتل الذي يستخدم في علاج الدودة على نطاق واسع واستخدم في التجربة كمادة مرجعية فقد أدى لوفاة الديدان بنسبة 100% عند كل التركيزات المستخدمة بعد 48 ساعة. أما بعد 24 ساعة فقد ماتت 75% من الديدان عند تركيزي 100 و 50 جزء في المليون و 50% من الديدان عند تركيز 25 جزء في المليون.

صابونيات الحبة السوداء و تانينات قشرة الرمان اختيرتا لدراسة توابع تأثيرهما على طبقة التيجيومنت المغلفة لدودة البلهارسيا باستخدام المجهر الالكتروني الماسح. اختيرت مجموعتي هذه المركبات نظرا لكفاءتها في قتل الدودة وتواجدها بكميات كبيرة في النباتات.

كلا مجموعتي المركبات أثرتا على طبقة التيجيومنت بشكل يتوقف على تركيزاتها المستخدمة. سببت الصابونيات تورم في التيجيومنت وتجعده والتحام الخطوط المرتفعة به وفقدان الدرنات السطحية للتيجيومنت بالإضافة على تقشره الذي نتج عنه قروح كبيرة على سطح الديدان سواء كانت مذكرة أو مؤنثة.

من ناحية أخرى تسببت تانينات قشرة ثمرة الرمان في تآكل طبقة التيجيومنت وإن كان ذلك ليس لدرجة إحداث قروح كما هو الحال في الصابونيات المستخلصة من الحبة السوداء. كما تسببت التانينات أيضا في إحداث تجعدات وتورم لسطح التيجيومنت وانفجار للدرنات في منطقة وسطها - كما نتجت من طبقة التيجيومنت كردات صغيرة بكثافة عالية أحاطت به. وقد نوقشت ظاهرة تدمير طبقة التيجيومنت وتأثيرها على مقدرة الدودة على مقاومة الجهاز المناعي وإنتاج الطاقة حيث أن التيجيومنت هو المكان الرئيسي لإنتاج الـ ATP المصدر الأساسي لطاقة الدودة المخترنة. كما أن تدمير التيجيومنت سيؤدي عدم قدرته على حماية أغشية الدودة من الشوارد المؤكسدة وبالتالي أكسدة الأغشية سوف يفقدها أيضا نفاذيتها الاختيارية.

هذا وقد تعرضت ممصات الدودة الفموية والباطنية لتشوهات وتدمير شديد عند تعرض الدودة لكل من الصابونيات والتانينات مما يؤدي لفقدان الدودة لمقدرتها على الإمساك بالأوعية الدموية وانجرافها مع الدم. بالإضافة لذلك فإن تدمير الممصات الفموية قد يفقد الدودة القدرة على التهام الدم وبالتالي حرمانها من غذائها الأساسي.

و ل قد أظهرت الاقطاعات المختلفة في الدودة والتي تمت صباغتها وفحصها بواسطة المجهر الضوئي وجود تدمير لطبقات الدودة التي توجد أسفل طبقة التجي ومننت وتشمل طبقات العضلات الاسطوانية والطولية حيث ظهرت بها بؤر متحللة وتجاويف فارغة.

هذا و قد تم إذابة بروتينات الديدان في محلول منظم و تم إمرارها في هلام أكريل غير متصل تحت تيار كهربائي (SDS-polyacrylamid gel electrophoresis) وكانت أهم التغيرات الملحوظة هي تناقص كميات بروتينات ذات أوزان 13, 59, 18 kDa مقارنة ببروتينات الديدان التي لم تتعرض للمركبات المستخدمة في حين أن البروتين 55 kDa زادت كميته. ومن المتوقع أن يكون لهذه البروتينات وظائف هامة للدودة تتعلق بحيويتها وطاقتها وشكلها الخارجي.

Abbreviation

LC _{100%}	Lethal concentration that kills 100%
LC _{50%}	Lethal concentration that kills 50%
mL	Milliliter
L.	Liter
mg	Milligram
hrs	Hours
g	Gram
ppm.	Part per million
°C	Temperature in degree centigrade
Sigma	A Chemical Company
DMSO	Dimethyl Sulfoxide
r	Correlation Coefficient
r ²	Square of correlation coefficient
kDa	Unit of weight of protein

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