In vitro Evaluation of Medicinal Plants' Crude Extract against Black rot (*Xanthomonas campestris* pv. *Campestris*) of Cabbage

Worke Gete[1] and Hagos Gereziher [2]

[1]Mettu University, Faculty of Natural and Computational Science, Department of Biology, Ethiopia
[2]Aksum University, Faculty of Natural and Computational Science, Department of Biology, Ethiopia
E-mail: woraget@gmail.com; Mobil : +251 923 958785

Abstract

Application of synthetic chemicals is one of the primary options for the control of the black rot disease. However, the environmental hazards and economic unfeasibility associated with them necessitate the search for relatively safe natural products with the ability to show antibacterial effects. With this view, an experiment was conducted to evaluate the effect of plant extracts on black rot of cabbage, Xanthomonas campestris pv. Campestris (XCC). XCC was isolated from infected cabbage leaves. The isolated pathogen grown in pure culture was maintained in Yeast Extract Dextrose Calcium Carbonate Agar (YDC) at 4 °C. The in vitro test was conducted to evaluate four methanol plant extract for their antibacterial effects against XCC using the paper disc technique. The minimum inhibitory concentration of methanol extracts of Phytolacca dodecandra, Ruta chalepensis, Ocimum lamiifolium and Vernonia amygdalina were 1.5, 2, 3 and 5%, respectively. In the in vitro trial, berries extracts of P. dodecandra at 10% concentration (10.88mm) showed the highest growth inhibitory effects as compared to other plant extracts, followed by R. chalepensis at 10% concentration (8.52mm). In general, this study demonstrated that plant extracts such as P. dodecandra, R. chalepensis, O. lamiifolium and V. amygdalina, inhibits the growth of XCC.

Keywords: Cabbage, plant extracts, *Xanthomonas campestris pv. Campestris*, In vitro, Black rot

Introduction

Background of the Study

Cabbage (*Brassica oleraceae* var. *capitata*) is one of the most important leafy vegetables grown and consumed worldwide (Talekar, 2000). It is an economically and nutritionally important crop produced in more than 90 countries of the globe and consumed widely among different society (Singh, 2009). Cabbage grown on 3.1 million hectares in 2007 (FAO, 2007), implies that it has wider cropland coverage globally. In Africa and Asia, the vegetable is very important crop

for smallholders through providing diversified food source and income that enable them to remain financially stable, especially in the rapidly growing peri-urban farming sectors. White-headed cabbage grew on 40,000 ha. in Kenya, Uganda and Tanzania; while Malawi, Zambia and Zimbabwe produced the crop on 10,000 hectares in 2006 (Zoss, 2006). The author also identified that Ethiopia and Cameroon produced white-headed cabbage on 4000 and 3000 hectares, respectively. Many of the African countries produced the white-headed cabbage for home consumption, and local and urban markets.

Black rot caused by *Xanthomonas campestris* pv. *campestris* (XCC) is the common disease that affect vegetables such as cabbage, swede, rape and mustard that are produced by smallholders in many of African and other developing countries (Anonymous, 2000). The disease was adequately described and identified by Pammel in 1895 in the US (Vicente, 2001). It is characterized by V-shaped, chlorotic to necrotic lesions at the margin of the diseased leaves and blackened vascular tissues. According to Massomo (2002) black rot caused substantial crop losses in all areas of Tanzania wherein cabbage grown commonly in the warm and humid seasons. In Ethiopia, Hussein (1989) identified that black rot of cabbage is the most destructive and prevalent disease causing a severe yield reduction mostly for smallholders that have traditional and nature dependent farming practices. Diseased crops have a poor market value and they are unsuitable for storage (Massomo, 2003). Since they quickly decayed after harvest, then there may be 100% yield losses for the producers. There is a frequent loss that range from quality deterioration to complete production loses. Disease control usually consists of using resistant cultivars and biological controls such as adoption of certified seeds, hot-water treatment of seeds, and application of antibiotics or protectant fungicides, crop rotation, control of weeds and insects, eradicating infected plants and debris from the cropland (Hildebrand, 1994). Additionally, several microbial antagonists have been identified to improve controlling alternatives against black rot disease. For instance, Dik (1991) found that yeasts have strong potential that could be used as cheap and easily accessible strategy of controlling agents, mainly because of its ability to compete for nutrients and colonization sites. Bacillus species including *B. cereus*, *B. megaterium* and *B. subtilis* tested to control the diseases through a variety of mechanisms such as nutrition competition, systemic resistance induction and antibiotic production (Leila, 2005). However, none of these biological agents were fully effective in controlling the disease and neither considered as commercial antigen nor available to cabbage producer smallholders in Ethiopia. Many of the smallholders in the country adopted synthesized chemicals in controlling black rot of cabbage (Kenny, 2008). *Xanthomonas campestris* populations may development of resistance due to continuous usage of synthesized chemicals (Cuthbertson and Murchie, 2005), which may create long lasting problem on the crop production. Additionally, the continuous usage of chemicals may generate environmental pollutions, and affects health condition of human and non-target organisms.

Having those facts in mind, it is imperative to explore cost effective and safe phytochemicals, which could be utilized to combat phytopathogens. Plants are known to possess various secondary

metabolites that have inhibitory effect on the growth of pathogens. Adoption of plant extracts is environmentally safe and cost effective way of managing crop diseases. This study helps in the commercialization and scaling up of the potential plant products about controlling the crop disease using plant extracts rather than cultural and chemical controls. Thus, this research gives insight regarding adoption of selected plant extracts to control black rot disease of cabbage caused by XCC considering sample plant species from Mettu district Illu Aba Bora zone south western, Ethiopia.

Materials and Methods

Study design

The experiment was arranged in completely randomized design (CRD) with three replications for each treatment and control.

Plant material collection and extraction

Four different species of medicinal plants (Table 1) collected from the surrounding area of Mettu town to test their crude extracts against *XCC*, the black rot pathogen of cabbage. The selected plant materials were collected from places in near-by distance to identify how accessible they are for smallholders. The collected plant materials then identified and authenticated by Addis Ababa herbarium experts and transported to Jimma University for extraction with a better technology. The air dried selected plant materials were ground until a fine powder is obtained that makes the extraction process easy. The powders were extracted at room temperature through dissolving 50gm plant powder in 250ml pure methanol and shake the mixture using a shaker for 2hours (Fazli *et al.*, 2012). The extracts were filtered through Whatman No.1 filter paper and then concentrated by evaporating methanol using a rotary evaporator. The extracts were stored in a refrigerator until it has been used in the anti-microbial bioassay.

Table 1: Description of the four selected medicinal plants

Scientific name	Common name		nt part used
Ocimum lamifolium	Demakese	*Demakese*	Leaf
Ruta chalepensis	Aleppo rue	*Tena Adam*	Leaf
Vernonia amygdelina	Bitter leaf	*Grawa*	Leaf and roots
Phytolacca dodecandra	Soap-berry plant	*Endod*	Berries

Isolation of the Target Organism

Xanthomonas campestris pv *campestris* (XCC) was isolated from infected cabbage leaves showing black rot symptoms. The leaves were thoroughly washed under tap water and small portions of the tissue from the advancing margin of a lesion cut out with a sterile scissor to reduce contamination. The cut portions was disinfected with 0.1% sodium hypochlorite for two minutes and then rinsed with sterile distilled water three times for two minutes (Arunakumara, 2006). The specimen was aseptically plated on the growth medium and incubated for 24hours at 29°C and then sub-cultured until pure isolates of *XCC* obtained (Fig.1). The isolate was then maintained at 4^0C on culture tube slants of

Yeast Extract Dextrose Calcium Carbonate Agar (YDC) and use as stock culture of the target organism (Lema *et al.*, 2012).

Minimum Inhibitory Concentration (MIC) and *In-vitro* Test against XCC

The MIC was determine by serial dilutions of methanol extracts of 1%, 1.5% and 5% concentration level, and the streptomycin at a recommended rate (0.66g/100ml) was inoculated with the test bacteria, XCC. The agar dilution assay and inoculation of the test bacteria was repeated three times to see reproducibility of the experiment. The lowest concentration the plant extract that inhibit visible growth of the bacteria after 24hours incubation considered to be the MIC (Vandepitte *et al.*, 2003). The results of MIC were given in mg/ml or percentage to make the explanation easy.

The crude methanol extracts of the four medicinal plants were reconstituted in Dimethyl Sulphoxide (DMSO) and tested in disc diffusion method, in which similar practice was done by (Bauer *et al.*, 1966). Nutrient agar medium of 28g/l was seeded with 100µl of inoculum (1×10^8 CFU/ml) and poured to 8cm diameter petri dish. Filter paper discs of about 6 mm diameter were placed in a beaker, covered with aluminum foil and sterilized in an oven at 150 °C for 1 hour. After that, they were impregnated with the test extracts by applying 10µl from the concentration (5% and10%) from each extract using a capillary pipette. After allowing the solvent to evaporate in a laboratory hood, the paper discs were placed on the agar medium seeded with the test microorganism (Tiwari *et al.*, 2004).

Additionally, a paper disc similarly handledwith methanol served as negative control and paper discs impregnated with commercial bactericide streptomycin at a recommended rate (0.66g/100ml) served as a positive control. Then the plates were incubated for 24 h at 29 °C to allow bacterial growth and zone of inhibition around the paper disc was measured in mm. The inhibition zone of pathogen with 0.00mm considered as the normal growth of the bacteria, However, when the value of inhibition zone greater than the indicated value considered as the growth of the bacteria inhibited. The experiment was laid out in completely randomized design (CRD) having 10 treatments (5% and 10% concentration for the four plants, and positive and negative control) with three replications.

Data collection and analysis

Data on minimum inhibitory concentration was obtained by serial dilutions of methanol extracts at a different concentration level of 1%, 1.5% and 5% and streptomycin at a recommended rate (0.66g/100ml). The lowest concentration that inhibits visible growth of the bacteria after 24 hours incubation was considered to be the MIC, and the inhibition zone of *XCC in vitro* test obtained from laboratory experiment measured in ruler in mm and scanned image editing was done through computer programming.

Data collected from laboratory experiments were transformed to the proper statistical software, SAS, to make the analysis scientific as much as possible. Analysis of variance was performed using the aforementioned software to check the variation created in the *in vitro* growth inhibition process. Mean comparison was done using Duncan's Multiple Range Test

(DMRT), which is a post hoc test to measure specific differences between pairs of means, at 5% level of significance.

Results and Discussion

Minimum Inhibitory Concentration (MIC)

This study showed the difference in the MIC of the four plant extracts on *XCC*. The MIC of *Phytolacca dodecandra* methanol extract against the pathogen was at 1.5%, and the normal growth of *XCC* observed below 1.5% methanol extract. Similar findings for the aqueous extract were reported by (Woldeamanuel *et al.*, 2005)' in which the MIC of *Phytolacca dodecandra* against the different Candida species of yeast was greater than 0.5%. Ameni and Tilahun, (2003) also showed that the MIC of the aqueous extract of *Phytolacca dodecandra* was at 1%. Additionally, *Ruta chalepensis* has the second best performing plant extract, which has MIC at 2%. Amomg the four plant extracts examined in this study *V. amygdalina* was least performing plant, in which it has MIC at 5%.

Table 2: Minimum inhibitory concentrations (MIC)

Plant species	MIC (%)
Phytolacca dodecandra	1.5
Ruta chalepensis	2
Ocimum lamiifolium	3
Vernonia amygdalina	5

In vitro Inhibitory effect of plant extracts towards *Xanthomonas campestris* pv. *Campestris*

In the present study the antibacterial activity against *XCC* of the four medicinal plant extracts was assessed at two different levels of concentrations (5% and 10%) in the laboratory using paper disc technique. All the tested plant extracts significantly ($p \leq 0.05$) inhibited growth of the pathogen at all concentration levels (Table 3). The SAS result presented in Table 3 revealed that there was statistically significant difference among MIC of the four plant extracts. The coefficients of each plant extract do not share common superscript alphabets, which imply that the inhibition zones were statistically different. Additionally, the coefficient of variation (CV (7.00 %)), which is below 10% showed there is overall statistically siginificant variation in the MIC of the plant extracts (Fig.3). Inhibition zone, diameter in mm, around the paper disc impregnated with treatments expressed as mean $\pm$ standard error (SE) means superscripted by the same letter (s) are not significantly different at $P \leq 0.05$ (Duncan's multiple range test (DMRT), coefficient of variation (CV) and standard error (SE)).

All the test plants extracts significantly ($p \leq 0.05$) inhibited the growth of the pathogen at all concentration levels (Table 3). For all plant extracts tested, growth inhibition of the pathogen showed increment at 10% concentration as compared to the 5% concentration. From the four tested plant extracts, extract of *P. dodecandra* at 10% showed the most potent antibacterial activity towards the pathogen with the highest inhibition zone with 10.88 mm relative to that of the negative control while the methanol extract of *V. amygdalina* at 10% inhibits the growth of the pathogen with inhibition zone of 3.06 mm. Extract of *V. amygdalina* also had the lowest value of inhibition zone with 0.54 mm whereas, extract of *P. dodecandra* showed the highest value of inhibitoin zone compared with negative control. The methanol

extract of *O. lamiifolium* showed moderate inhibition effects at 5% and 10% concentration level with 2.40 mm and 5.08 mm respectively compared with negative control. This result in accordance with Tadeg *et al.*, (2005) also used *P. dodecandra* among other plant species to evaluate their efficacy against some human bacterial and fungal strains causing skin infections and the plant was one of those that showed antimicrobial property against at least one microbial strain.

According to Ahmed-Emam *et al.* (2010), *R. chalepensis* also exhibited activity against phyto-pathogenic fungi like *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Fusarium solani*, which cause root-rot and wilt diseases in several economically important food crops such as potato, sugar beet and tomato. This may be due to the presence of alkaloids, coumarins and flavonoids (Farag, *et al.*, 2005) which play an important role in the rue plant's chemical defense against plant pathogens. Another study showed that the extract of this plant exhibited very strong antibacterial effect against Gram positive cocci and Gram negative rod-shaped bacteria like *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus luteus*, and *Escherichia coli* and *Enterococcus faecium*.

Table 3: In vitro test for growth inhibition of *XCC*

Plant species	Concentration (%)	Inhibition zone (mm)
Phytolacca dodecandra	5	4.78^{e}
	10	10.88^{b}
Ruta chalepensis	5	3.49^{f}
	10	8.52^{c}
Ocimum lamiifolium	5	2.40^{g}
	10	5.08^{d}
Vernonia amygdalina	5	0.54^{h}
	10	3.06^{gf}
Streptomycin		36.68^{a}
Methanol		0.00^{h}
CV (%)		7.00

Moreover, the growth inhibiting activity was more potent against Gram positive than Gram negative bacteria (Enis- Ben-Bnina *et al.*, 2010). Similar report *in vitro* research was done by agar disc diffusion method on the aqueous, ethanol and methanol extracts of *O. lamiifolium* revealed that the three extracts have antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Shigella boydii* (Damtie and Mekonnen, 2015; Gebrehiwot and Unakal, 2013).

Finally, the negative control of the test process was DMSO and it did not exhibit any antibacterial activity (0.00mm) against *XCC*. Streptomycin (applied at 0.66g/100ml) was used as a positive control and completely inhibited the bacterial growth (36.68mm).

Conclusions and Recommendation

Conclusion

All plant extracts tested in this study show significant inhibition zone against growth of the pathogen, XCC, at all concentration levels. *Phytolacca dodecandra*, with many antimicrobial properties, is the best plant species, which have proved superiority *in vitro* effect against XCC, followed by leaf extracts of *Ruta chalepensis*.

The methanol extract of *Ocimum lamiifolium* showed moderate inhibition zone at both 5% and 10% concentration level compared with the postive control.

Generally, this study demonstrated that the plant extracts considered such as *Phytolacca dodecandra, Ruta chalepensis, Ocimum lamiifolium* and *Vernonia amygdalina*, inhibited the growth of *XCC* differently as per their active biological constituents.

Recommendation

The antimicrobial activities of the four medicinal plants were tested against *XCC* in the laboratory work only, so further study may be needed to verify antimicrobial effectiveness of the four plants in field condition. Additionally, there is a waiting research task that is evaluating medicinally importance of those plant species with different types of solvents.

References

Ahmed E, Mohamed E. and Medhat E., 2010 A new furoquinoline alkaloid with antifungal activity from the leaves of *Ruta chalepensis* L. *Drug Discoveries and Therapeutics.* 4(6):399-404.

Ameni, G., and Tilahun, G., 2003. Preliminary laboratory and field evaluation of Endod for treatment of epizootic lymphangitis. *Bull Anim Health Prod Afr.*, 51:153–160.

Anonymous, 2000. Plant protection manual for selected vegetables: French beans, Brassicas and tomatoes. GTZ/ICIPE Nairobi, Kenya.

Arunakumara, K.T., 2006. Studies on *Alternaria solani* (Ellis and Martin) Jones and Grout Causing Early Blilght of Tomato. Msc thesis Department of Plant Pathology college of Agriculture, Dharwad University of Agricultural Sciences, Dharwad – 580 005.

Bauer A.W., Kirby W.M., Sherries J.C. and Tuck M. 1966. Antibiotic susceptibility testing by a standardized disc diffusion method, Ameri. *Journal of Clinical Pathology*, 45: 493-496.

Cuthbertson A.G.S. and Murchie A.K., 2005. Economic spray thresholds in need of revision in Northern Irish Bramley orchards. *Bio. News*, 32: 19.

Damtie D. and Mekonnen Y., 2015. In vitro evaluation of the antibacterial activities of the methanol, aqueous and n-hexane extracts of *Ocimum lamiifolium* from Ethiopia. *African Journal of Pharmacy Pharmacology* 9: 91-97.

Dik A.J., Fokkema N.J. and Van pelt J.A., 1991. Consumption of aphid

honeydew, a wheat yield reducing factor, by phyllosphere yeasts under field conditions. Neth. *Journal of Plant Pathology* 97:209- 232.

Doughari J. H., Elmahmood A.M. and Manzara, S. 2007. Studies on the antibacterial activity of root extracts of *Carica papaya* L. African. *Journal of Microbiology Research*; 37- 41.

Enis B.B., Saoussen H.M., Daamiiremadi H.B., and Jannet Z.M., 2012. Chemical composition and antimicrobial effects of Tunisian *Ruta chalepensis* L. essential Oils. *International Journal of Agriculture and Crop Science* 4: 709-712.

FAO, 2007. Food and Agriculture Organization, United Nations. http://faostat. fao.org.

FAO (Food and Agriculture Organization) of the UN, 2010. Brassicas Today. (http://faostat.fao.org/site/567/default.asp#ancor, Accessed 15 November 2010).

Farag M.M., Emam A.M. and Mahmoud M.E., 2005. *In vitro* evaluation of the antioxidant effect of some botanical ethanolic extracts and isolation of the antioxidant constituent(s) from *Ruta chalepensis* Leaves. *Journal of Agricultural Research Development*, 19:178-190.

Fazli K., Zafar I., Ayub K., Zakiullah F.N. and Muhammad S.K., 2012 .Validation of some of the ethno Pharmacological uses of *Xanthium strumarium* and *Duchesnea. indica Pak. Journal of Botany*, 44(4): 1199-1201

Gebreniwot A and Unakal C.G., 2013. Effect of aqueous and ethanol extracts of *Ocimum lamiifolium* and Amaranthus dubius against bacteria isolated from clinical specimen. *International Journal of Pharmacy and International Research* 3: 10-14

Hildebrand P.D., Howard R.J., Garlan D. and Seaman W.L., 1994. Black rot. In diseases and pests of vegetable crops in Canada: An Illustrated Compendium. The Canadian Phytopathological society and The Entomological Society of Canada, Ottawa. pp. 93-94.

Kenny S., Paul B. and Julie B., 2008. Black Rot of Crucifers. Plant Pathology Fact Sheet UK Cooperative Extension Service.

Lambert J.D., Temmink J.H., Maquis J., Parkhurt R.M., Lugt C.B., Lemmich E., Wolde Y.L. and Desavingny D. 1991. Endod: Safety Evaluation of a Plant Molluscicide, *Regul. Toxicol. Pharmacol*, 14(2): 189-201.

Leila M., Rosa de L., Ramos M. and Ana-Maria S.M., 2005. Brazilian Archives of Biology and Technology. *An international journal*. 48: 23-29.

Lema M., Velasco P., Soengas P., Francisco M., Cartea E.M. 2012. Screening for resistance to black rot in *Brassica oleracea* crops. Plant Breeding 131, 607–613.

Massomo S.M.S. 2002. Black rot of cabbage in Tanzania: Characterization of *Xanthomonas campestris* pv. campestris. and disease management strategies. The Royal Veterinary and agricultural University, Copenhagen. Denmark.

Massomo S.M., Hanne N., Mansfieldgiese K., Mabagala R.B., Hockenhull J. and Mortensen C.N., 2003. Identification and characterization of *Xanthomonas campestris* pv. *campestris* from Tanzania by pthogenicity tests, Biology, Eric- and Box-PCR and fatty acid methyl ester analysis. *European Journal of Plant Pathology*. 109: 775–789.

Singh B.K., Sharma S.R. and Singh B., 2009. Heterosis for mineral elements in single cross-hybrids of cabbage

(*Brassica oleracea* L. var. *capitata*). *Sci. Hortic.*, 122:32–36.

Tadeg H., Mohammed E., Asres K. and Gebre-Mariam T., 2005. Antimicrobial activities of some selected traditional Ethiopian medicinal plants used in the treatment of skin disorders. *Journal of Ethnopharmacol.*, 100: 168-75.

Talekar N.S., 2000. Chinese cabbage. Proceedings of the 1st International symposium on Chinese cabbages. AVRDC, pp 67-69.

Tiwari R.K.S., Chandravanshi S.S. and Ojha B.M. 2004. Evaluation of some medicinal plant species for their antibacterial activity against *Xanthomonas campestris* pv. *campestris*, the black rot pathogen of cabbage. *Indian Phytopath.* 57 (3): 308-31

Vandepitte J., Verhaegen J., Engbaek K., Rhoener P., Piot P. and Heuk C.C., 2003. General principle of antimicrobial susceptibility testing. Basic Laboratory procedures in clinical bacteriology, 2nd Ed. WHO, Geneva. Pp 133-136.

Woldeamanuel Y., Abate G. and Chryssanthou E., 2005. *In vitro* Activity of *Phytolacca dodecandra* (Endod) againest dermatophytes. *Ethiopian Medical Journal.* 43(1): 31-34

WHO (World Health Organization), 2002. Traditional medicine: Growing needs and potential. Policy perspectives on medicines, Geneva, P. 1-6.

Zoss M. 2006. Vegetable value chain in Northern Tanzania: Mode of governance, collective actions and facilitator's intervention Swiss Federal Institute of Technology Zurich.