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Full Length Research Paper

In vitro antibacterial activity of *Tagetes minuta* and *Capsicum frutescens* extracts against *pectobacterium carotovorum*

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Pectobacterium carotovorum **bacteria cause soft rot and vascular wilt of vegetables in the field and post harvest decay. The aim of this study was to determine *in vitro* antibacterial activity of *Tagetes minuta* and *Capsicum frutescens* extracts against *Pectobacterium carotovorum.* Aqueous extracts of *T. minuta* and *C. frutescens* were tested against *Pectobacterium* by modified disc diffusion method on agar plates and potato chips assay. Streptomycin sulphate and distilled water were the positive and the negative controls respectively. The experiment was set up in a completely randomized design. Data was collected on zone of growth inhibition, number of days to total tissue maceration and %weight loss of potato chips due to tissue maceration. The three concentrations of *T. minuta* (40%, 30% and 20%) recorded zones of inhibition of 7.167mm, 6.667mm and 6.1mm; and streptomycin sulphate recorded 8.83mm which were significantly different from *C. frutescens* and distilled water that recorded 0.00mm. In the potato chips assay, *T. minuta* and streptomycin sulphate showed a significant difference in the number of days (9days) to total tissue maceration and % weight loss from *C. frutescens* and water that took only 5days for the potato chips to be totally macerated. It can be concluded that extracts from *T. minuta* have antibacterial activity against *Pectobacterium carotovorum*.**

Key words*: In vitro*, growth inhibition, *Pectobacterium carotovorum*, *Tagetes minuta*, *Capsicum frutescen*s.

INTRODUCTION

Pectobacterium carotovorum bacteria have a wide host range causing soft rot diseases in vegetable crops including carrots, cucumbers, potatoes, tomatoes and cabbages (Abeer *et al*., 2014, Asma *et al*., 2014). These bacteria that produce extracellular pectic enzymes such as pectate lyase isozymes, cellulase, polygalacturonase and protease, are the primary cause of soft rot and vascular wilt of vegetable crops in the field and decay in the post harvest chain (Rayavarapu *et al.,* 2014). *Pectobacterium carotovorum* subsp, *carotovorum* causes bacterial soft rot in tubers in the field, during transit and in storage. It also causes hollow stem and blackleg; a blackening of the stem base of potato plants, which may originate from the seed tuber (Pérombelon and Kelman 1987, Zebene *et al.,* 2014).

As noted by Czajkowski *et al.,* (2011), blackleg of potato is characterized by lower stem blackening, yellowing of foliage and death of the emerged plant in the field and persists on older plants causing infection of young potato tubers. This agrees with Mario *et al*., (2005) who also noted that the diseased plants show long dark-brown/black longitudinal stem lesions, soft stem rot, pith breakdown, hollow stems and vascular tissue discoloration in tubers. In tubers, *Pectobacterium* infection takes place at wound sites, through lenticels and the stolon ends under wet conditions. The symptoms of attack in tubers are wet, foul smelling water-soaked

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lesions due to tissue maceration that may engulf the entire tuber (Acero-ortega *et al.,* 2003, Czajkowski *et al.,* 2011).

Chemical control of the disease relies on use of copper-based compounds and antibiotics like streptomycin but as Kotan *et al*., (2007) noted, the indiscriminate use of such agrochemicals leads to degradation of the ecosystem, induce pathogen resistance and exert negative impacts on both animals and humans. As such, use of synthetic chemicals is restricted or even forbidden in many countries as noted by Gracia-Garza *et al*., (2002)**.** In addition, Muhammad *et al.,* (2014) observes that the escalating emergence of antibiotic resistance to synthetic chemicals (like Streptocycline) has drawn the attention of researchers towards medicinal plants in search of new, less toxic and effective formulations against microbes. This is supported by Nwachukwu and Umechuruba, (2001) who noted that in recent years much attention has been given to nonchemical systems to protect crops against pathogens. Plant extracts have a significant role in the inhibition of pathogens and therefore offer alternative methods of pest control to be incorporated in integrated pest management (IPM) systems to reduce over dependence on synthetic pesticides.

Natural products from plants contain antimicrobial compounds (Ngadze, 2014) that inhibit peptidoglycan synthesis, damage microbial membrane structures, modify bacterial membrane surface hydrophobicity and interfere with quorum sensing of the pathogens (Shayan and Saeidi, 2013). Secondary plant products (flavonoids, steroidal alkaloids and saponins) show antibacterial activity against plant pathogens **(**Ogowike *et al.,* 2013) and antimicrobial activity of polyphenols present in vegetable, food and medicinal plants have been investigated against microorganisms (Patricia *et al.,* 2013)**.**

Some chilli species contain *capsaicinoids* which have anti-bacterial effects in human bacterial pathogens like *Staphyllococcus sp*., *Escherichia coli*, *Bacillus aureus* and *Bacillus subtilis (*Soetarno *et al., 1997).* Growth of *Pectobacterium carotovorum* was inhibited by extracts from three varieties of *Capsicum annuum* and by some of the compounds found in the extracts, like *meta*-coumaric and *trans*-cinnamic acids (Acero-ortega *et al.,* 2003). *Tagetes minuta* contains essential oils (dihydrotagetone, β-ocimene, terpinolene, piperitone, β-caryophyllene); the major terpenes present in the *Tagetes* plants (Ester *et al.*, 2008, Supradip *et al*., 2012). These oils have antifungal activity against *Aspergillus niger*, *Aspergillus flavus, Rhizoctonia solani, Sclerotinia sclerotiorum* and *Sclerotium rolfsii (*Maria *et al.,* 2006). Generally, not much has been reported on the effect of *Tagetes minuta* on bacterial growth inhibition. In light of these, we experimented with *Capsicum frutescens* fruit and *Tagetes minuta* extracts on *in vitro* growth inhibition of *Pectobacterium carotovorum*. Aqueous crude extracts of *Capsicum frutescens* and *Tagetes minuta* were used to determine *in vitro* growth inhibition of *Pectobacterium carotovorum* bacteria.

MATERIALS AND METHODS

Research site

The experiment was conducted in the Horticulture Research and Teaching Laboratory at Egerton University, Njoro, Kenya. The laboratory lies at a latitude of 0º 23' South, longitude 35º35' East, altitude of approximately 2,238 meters above sea level in the Lower Highland 3 (LH3) agroecological zone (Jaetzold et al., 2005). The laboratory's average maximum and minimum temperatures were 19ºC to 22ºC and 5ºC to 8ºC, respectively with about 75% RH.

Plant materials

Tagetes minuta plant material growing as weeds was collected from the farm fields at Egerton University, Nakuru, Kenya. Red ripe *Capsicum frutescens* fruits were purchased from Nakuru farmers' market. The materials were positively identified and confirmed by a botanist at the Department of Biological Sciences, Egerton University.

Preparation of extracts

The leaves and stems of *Tagetes minuta*; and *Capsicum frutescens* fruits were used to produce the two crude extracts that were evaluated. The materials were air dried under shade for three weeks then grounded using an electric grinder (SB-808 by SAYONA PPS). The two extracts were separately homogenized in distilled water in the ratio of 1:10 (W: V) and steeped for 12 hours at 30°C on a rotary shaker (THZ-C-1 Hangzhou, China). The materials were filtered through a muslin cloth and centrifuged (KUBOTA 6800, Japan) at 5,000g for 15 minutes. The supernatants were collected and concentrated in a water bath at 70°C to make the final volume, one fifth of the original volume (which served as 100% concentration of each extract). The two extracts at 100% concentration were then stored at 4°C until evaluation (Jigna *et al.,* 2005). The extracts were each diluted to 40%, 30% and 20% (V:V) concentration by mixing the 100% concentration extracts with distilled water at the ratios; 1:2.5, 3:7 and 1:5 (extract: water). The diluted extracts were used for evaluation in the modified disc diffusion method.

Preparation of the agar plates and bacterial nutrient broth

Nutrient agar and nutrient broth were each prepared accord-

ing to the manufacturer's instructions then sterilized for 15 minutes at 121°C. After cooling to 45°C, nutrient agar was poured aseptically into sterilized petri plates of diameter 9 cm (10ml). The media was allowed to solidify in petri plates for about an hour in a laminar flow hood, and then placed in an inverted position to avoid evaporation of water from the medium within the plates. After 24 hours, uncontaminated plates were used to culture the bacteria. The nutrient broth in glass bottles was later used for incubation of the bacteria in liquid suspension form.

Isolation of the pathogen

Pectobacterium carotovorum bacteria were obtained from naturally infected potato tubers. After surface sterilization with 70% ethanol solution and washing three times in sterile water, the potato sections were grounded in small volumes of sterile water to obtain a potato paste containing the bacteria. A sterile loop was used to pick and streak the bacteria onto nutrient agar plates which were then incubated at 22°C for three days according to Ni *et al*., (2010) to produce single, round, convex, creamy-translucent, raised and shiny colonies on the nutrient agar (figure 1). The colonies were sub-cultured 3times to obtain pure cultures which were maintained and covered for subsequent uses (Perombelon and Wolf, 1998).

The top of single and well-isolated colonies was picked with a sterile loop and inoculated into the 250 ml of nutrient broth. The broth culture was then incubated for 12 hours to obtain young cultures. The turbidity of actively growing broth cultures was then adjusted to a 0.5 McFarland standard comparable to a bacterial suspension of 1×10^8 CFUml^{-1}. The bacterial suspension was later used to inoculate the sterilized agar plates and for the potato chips assay.

Inoculation of the agar plates

A sterile cotton wool swab was used to inoculate the bacteria from the nutrient broth culture onto the surface of the nutrient agar plates. This was swabbed over the entire surface of the nutrient agar medium in the petri plates and then allowed to dry at room temperature in the laminar flow hood.

A micro-pipette was used to place 4 drops of the three concentrations (40%, 30% and 20%) of each of the plant extracts, streptomycin sulphate (100ppm) and sterile water on the inoculated agar plates. The treatments were applied by placing four drops of each extract for the three concentrations, water and streptomycin onto inoculated agar plates using a sterile micro-pipette. Each treatment was applied in separate agar plates with the four drops serving as replications. The plates were kept in the laminar flow hood for 1 hour to allow for diffusion of the test material into the medium then moved to the incubator at 22°C. After 12 hours, the plates were examined for bacterial growth inhibition and the inhibition zone diameter was measured in millimeters (mm) using a pair of calipers and recorded as a measure of the antibacterial activity.

Potato chips assay

A second experiment was set up to test the number of days it would take for complete tissue maceration of potato chips treated with the different concentrations of the plant extracts. Potato chips weighing 5g were immersed in 10ml of distilled water in test tubes with two drops of the bacteria from the nutrient broth suspension equivalent to 100 μl of 10^8 CFU ml^{-1}. The treatments were then applied by placing three drops, two drops and one drop of the 100% *Tagetes minuta* and 100% *Capsicum frutescens* extracts respectively into the test tubes containing the potato chips in the 10ml bacterial suspension. Three drops of 100ppm streptomycin sulphate and distilled water were also used as positive and negative controls respectively. The experiment was set up in a completely randomized design with each of the treatments replicated three times. These were incubated at 22°C then checked for tissue maceration and recorded in number of days taken for tissues to be completely macerated. Tissues were considered to be completely macerated when there was no visible potato chips in the test tube and everything was observed floating on the test solution in the test tube.

Percent weight loss due to tissue maceration

A third experiment was set up to test the effect of the different concentrations of *Tagetes minuta* extracts on the weight loss due to tissue maceration of potato chips. Potato chips weighing 5g were immersed in 10ml of each of the test material (40%, 30% and 20% *T. minuta*, distilled water and 100ppm streptomycin sulphate) in test tubes with 100 μl of 10^8 CFU ml^{-1} of the bacteria. The experiment was set up in a completely randomized design and each of the treatments was replicated three times. These were incubated at 22°C then checked for weight loss due to tissue maceration by washing off the macerated tissues and recorded as % weight loss from the initial 5g at two days' interval until all tissues were completely macerated (when no more solid potato chips were left for weighing).

RESULTS

Data collection and analysis

The data collected was subjected to Analysis of Variance (ANOVA), using Genstat Edition 4 and means with significant differences separated using the Tukey's Hone-

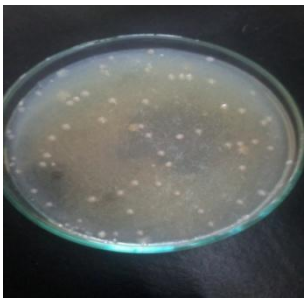

Figure 1. Bacterial colonies on agar.

stly Significant Different Test at P ≤0.05.

***In vitro* bacterial growth inhibition**: In this study antibacterial activity of *Tagetes minuta* and *Capsicum frutescens* extracts against *Pectobacterium carotovorum* bacteria were evaluated by a modified disc diffusion method. The diameter of the zone of inhibition produced by each of the treatments was measured in mm using a pair of calipers and recorded as a measure of the antibacterial activity after 12 hours of incubation at 22°C.The results of the *in vitro* tests are expressed in mm of the zone of inhibition as shown in (table 1).

SM: 100 ppm Streptomycin sulphate, M1:40% *T*. *minuta*, M2: 30%*T*. *minuta*, M3: 20% *T*. *minuta*, C1: 40% *C*. *frutescens*, C2: 30%*C*. *frutescens*, C3; 20%, W: Distilled water

There was a significant difference in the antibacterial activity of the plant extracts against *Pectobacterium* bacteria whereby *T. minuta* (b), showed Zone of Inhibition of 7.167mm, 6.667mm and 6.1mm (table 1) for the three concentrations (40%, 30% and 20% respectively) that was close to that of streptomycin sulphate (a), that had an inhibition zone of 8.83mm. On the other hand, *Capsicum frutescens* extracts (c) and water showed 0.00mm inhibition on the growth of the *Pectobacterium* bacterial colonies.

Days to total tissue maceration of potato chips: The number of days it took for the potato tissues to get totally macerated was recorded for each of the treatments by observing through the glass (test tube) until when the tissues floated on the surface of the solution with no visible potato chips (figure 2). There was a significant difference in the number of days to total tissue maceration. The potato chips that were treated with *T. minuta* extract took the longest time (9days) after Streptomycin sulphate (10 days) for the tissues to be completely macerated as shown in table 2 and figure 3. On the other hand, the potato chips that were treated with *C. frutescens* fruit extracts got completely macerated by the 5th day just the same as those treated with water. By the 8th day, there was only a floating material in the test tubes with *C. frutescens* extracts and water but the test tubes with *T. minuta* extracts and streptomycin sulphate still had some potato chips visibly seen in the test tubes (figure 3).

SM: Three drops Streptomycin sulphate, M1: Three drops *T. minuta*, M2: Two drops *T. minuta*, M3: One drop *T. minuta,* C1: Three drops *C. frutescens*, C2:Two drops *C. frutescens*, C3:One drop *C. frutescens*, W:Water

Percent weight loss due to tissue maceration: In this experiment, the effect of *T. minuta* on % weight loss on the potato chips was tested against that of streptomycin and water at two day intervals. Weight loss in potato chips due to tissue maceration was measured by washing off the rotten tissues then subtracting from the original weight of 5g and recorded as % weight loss. There was a significant difference in %weight loss due to tissue maceration whereby the potato chips that were treated with water had the highest % weight loss while those treated with *T.minuta* had the lowest %weight loss in all the three concentrations as shown in (figure 4) below. Increasing the concentration of *T.minuta*, reduced % weight loss due to tissue maceration; the potato chips treated with 40% marigold had the lowest % weight loss followed by those treated with 30%, and those treated with 20% (figure 4).

SM: 100ppm Streptomycin sulphate, M1:40% *T*. *minuta*, M2: 30%*T*. *minuta*, M3: 20% *T*. *minuta*, W: Distillled water.

DISCUSSION

***In vitro* bacterial growth inhibition**: In comparison to conventional antibiotics (100ppm Streptomycin sulphate) used in this study, *Tagetes minuta* extracts at 40%, 30% and 20% concentration (table 1) showed antibacterial activity against the *Pectobacterium carotovorum* bacteria *in vitro*. The results agree with Ogowike *et al.,* (2013) who reported that secondary plant product like flavonoids, steroidal alkaloids and saponins show antibacterial activity against plant pathogenic bacteria. The secondary products in *T*. *minuta* extracts may have influenced antimicrobial activity on *Pectobacterium carotovorum* by inhibiting the peptidoglycan synthesis, damaged microbial membrane structures and modified the bacterial membrane surface hydrophobicity of the *Pectobacterium carotovorum* as indicated by Shayan and Saeidi, (2013).

Dagmar *et al*., (2008) reports *in vitro* effectiveness of

Table 1. Minimum inhibition zone on bacterial growth (mm).

Treatment	Mean
SM	8.833a
M1	7.167b
M2	6.667b
M3	6.100c
C1	1.000d
C2	1.000d
C3	1.000d
W	1.000d

Means followed by the same letter are not significantly different at P≤0.05.

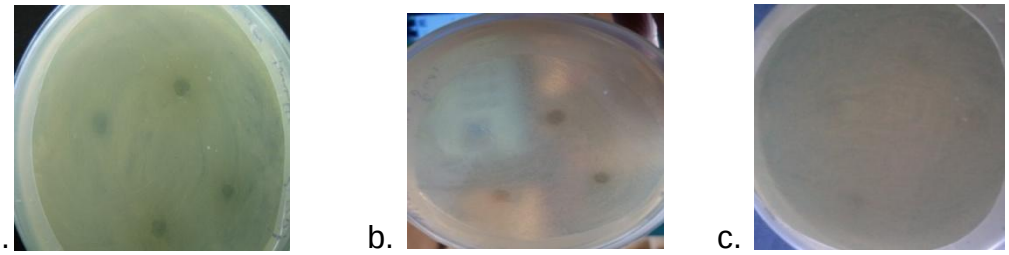

Figure 2. Antibacterial activity of Streptomycin sulphate (a), and *Tagetes minuta* (b) and *Capsicum frutescens* (c) by modified disc assay against *Pectobacterium carotovora*.

Table 2. Number of days to total tissue maceration.

Treatment	**Number of Days**
SM	10a
M1	9a
M2	9a
M3	7b
C1	5.33bc
C2	5d
C3	4.67d
W	5d

Means followed by the same letter are not significantly different at P≤ 0.05.

Tagetes bipinata against *Clavibacter michiganensis* which causes ring rot in potato and bacterial wilt in Lucerne. The essential oils in *Tagetes bipinata* which had inhibitory effects on the *C*. *michiganensis* may have been present in *T*. *minuta* causing the same inhibitory effects on *Pectobacterium carotovorum* in the current experiment.

Reports by Bhat *et al*., (2012) indicate that the phenolic compounds in *T*. *minuta* are capable of dissolving within the bacterial membrane and thus penetrating inside the

a.  b.  c.  d. 

Figure 3. Extent of tissue maceration by the 9th day after incubation with *Tagetes minuta* (a), Streptomycin sulphate (b), *Capsicum frutescens* (c) and water (d) respectively.

Table 3. Percent weight loss after 2-11 DAI (Days after inoculation).

Treatment	2DAI	5DAI	7DAI	9DAI	11DAI
M1	10.267	11.80	16.60	27.07	50.60
M2	6.667	13.60	32.87	50.13	78.80
M3	7.933	22.80	40.47	62.80	83.87
SM	3.00	36.87	58.93	80.40	93.97
H	20.800	53.73	74.27	92.40	100

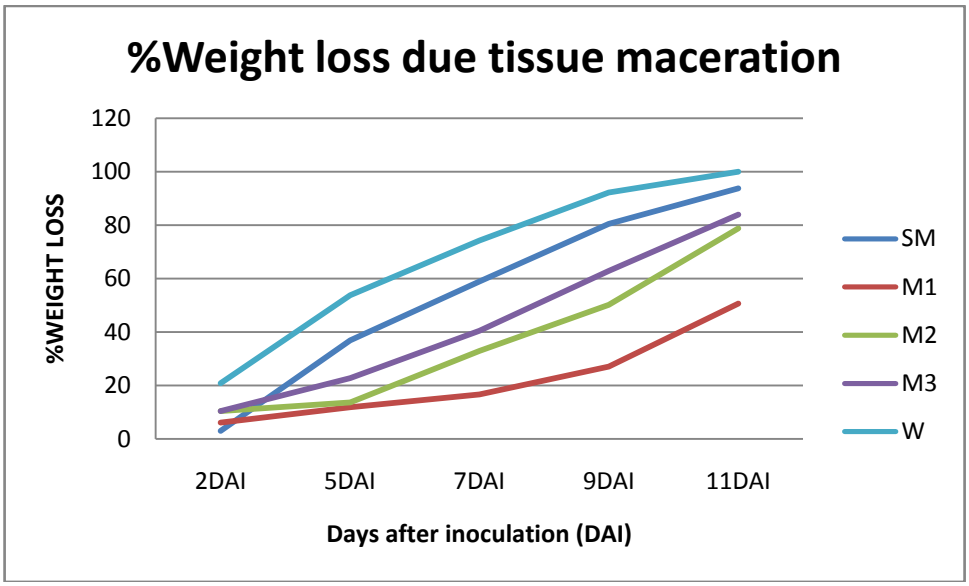

Figure 4. Weight loss due to tissue maceration.

cell, where they interact with cellular metabolic mechanisms thereby killing the bacteria as confirmed in our study.

Capsicum frutescens extracts showed no activity against *Pectobacterium carotovorum* contrary to the findings by Acero-ortega *et al.,* (2003) who reports that growth of *Erwinia carotovora (Pectobacterium carotovorum)* is inhibited by extracts from the three varieties of *Capsicum annuum* and compounds found in the extracts, like *meta*-coumaric and *trans*-cinnamic acids. However, in the same experiment, it is reported that other compounds; Capsaicin and dihydrocapsaicin compounds do not affect the growth of the same bacterium which agrees with our findings.

The inactivity of *Capsicum* against *Pectobacterium carotovorum* in the current research may be attributed to the use of a different species of *Capsicum*. *Capsicum annuum* was used by Acero-ortega et al., (2003) while in

the current study, *Capsicum frutescens* was used which may have had differences in the amount of the inhibitory compounds in each of them. In the current study, the results may have also been different because the extracts were obtained using the aqueous extraction instead of the Soxhlet extraction method (using Isopropyl alcohol) used by Acero-ortega *et al*., (2003). However, the current results are in agreement with Nagoth *et al., (*2013*)* who reports that acetonitrile and acetone extracts macerated by the 5th day. In an experiment by Asma *et al.,* (2015), five isolates of *Pectobacterium* (*Erwinia*) evaluated for aggressiveness on tomato fruits, and chilli isolate are found to be the most aggressive followed by tomato and potato isolates producing 22.3 mm, 7.9 mm, and 7.8 mm diameter of soft rot lesions, on the fruits respectively. This shows that the *Pectobacterium carotovorum* infects *Capsicum sp* and as such the *Capsicum frutescens* extracts in the current experiment may have acted as a nutrient instead of killing the bacteria. Work by Zia *et al.,* (2011), Hafiz *et al.,* (2014) where *Capsicum chinense* is used as an enrichment host for *Erwinia* during isolation of *Erwinia* bacteria before culture also supports this theory.

Percent weight loss due to tissue maceration: In this experiment, the three concentrations (40%, 30% and 20%) of *T*. *minuta* were evaluated for percent weight loss due to tissue maceration by the *Pectobacterium* bacteria. *T. minuta* had the lowest percent weight loss of the potato chips due to tissue maceration by *Pectobacterium carotovorum* compared to water and even streptomycin sulphate (table 3). The result corroborate with the results obtained by Bhat *et al.,* (2012) who observe that the inhibitory effect of flower extracts from *T.erecta* and *T*. *patula* on *S*. *aureus*, *S*. *epidermidis* and *E*. *coli* is higher than that of streptomycin. Kotan *et al., (*2007) also reports a significant antibacterial activity by the essential oils of *T. canoviridis, S. hortiensis, M. officinalis* ssp. *inodora, H. pilicatum, T. haussknechtii and T. sipyleus* on inhibition of *X. axonopodis* pv. *Vesicatoria* which proves to be stronger than the standard antibiotic (Streptocycline) used. Findings by Muhammad *et al.,* (2014) too indicate that in comparison to the antibiotics used in their study, the plant extracts are far more active against the test bacterial strains.

A study by Hajhamed *et al*., (2007), show that *Tagetes minuta* induces systemic resistance against *Pectobacterium* or even suppresses enzyme production which decreases the maceration of the tissues; while Reena *et al.,* (2012), Lubna and Tahir (2012), show that *Tagetes sp.* contains secondary metabolites including flavonoids and terpenes that have pharmacological and antibacterial activity. This is in agreement with the current results that showed suppressed activity of *Pectobacterium carovorum* in the potato chips assay.

Ogoiwike *et al.,* (2013) indicates that the synthesis and modification of the plant products in plant tissues are controlled by different enzymes like glucosyltransferases. from *Capsicum chinense* were found to be ineffective against *E.coli* and *Erwinia sp.*

Days to total tissue maceration of potato chips: Potato chips treated with *T*. *minuta* lasted 9 days before total maceration while those treated with streptomycin lasted 10days, implying that *T*. *minuta* had almost the same antibacterial effects as streptomycin sulphate. On the other hand, potato chips that were treated with *Capsicum frutescens* and distilled water were completely In addition, Czajkowski *et al.,* (2011) found improved resistance of potato tubers against *Pectobacterium carovorum* in *in vitro* experiments where the resistance to soft rot was two-fold higher in transgenic lines than in non-transformed control tubers. In the current study, the *T.minuta* may have induced resistance in the potato chips to the *Pectobacterium carotovorum* which lost only slightly above 50% of the weight of the potato chips compared to the chips that were treated with sterile water that had lost all the tissue to tissue maceration by the 9th day (figure 4).

Studies by Mohsen *et al.,* (2014) show that *Tagetes minuta* has been used as a food colorant in foods such as pasta, vegetable oil, margarine, mayonnaises, salad dressing, baked goods, confectionery, dairy products, ice cream, yogurt, citrus juice, mustard and as colorant in poultry feed because of the rich orange-yellow carotenoid. Based on the present results and others *T*. *minuta* can be used to extend the shelf life of potato chips under ambient temperature conditions and still be used for human consumption.

CONCLUSION

Results obtained in this study are in conformity with reports that *Tagetes minuta* has antimicrobial activity against various microbes indicating that the plant has potential for incorporation into the integrated pest management systems in the management of soft rot caused by *Pectobacterium carotovorum* as an alternative pesticide instead of using the synthetic chemicals. However, further research is required to determine the active ingredients in the *T. minuta* extracts that actually inhibits the growth of the bacteria.

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