Herbal extract targets in *Leishmania tropica*

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Abstract The present study aims to investigate the effect of some herbal extract such as phenolic compounds on the viability of *Leishmania tropica* promastigotes in vitro. Four tested chemical agents (caffeic acid (CA), ferulic acid (FA), syringic acid (SA) and 4-hydroxybenzoic acid (4-HBA)) were used in this study. The viability of *Leishmania tropica* promastigotes was investigated under five different concentrations (10, 15, 20, 25 and 30 mg/ml) of each agent after (72 h). CA was the most active agent on the promastigotes viability after 72 h exposure to 30 mg/ml concentration so that the parasitocidal effect reach (53×10^4) promastigote/ml. FA is the second agent in parasitocidal effect that parasitocidal effect reach to (50×10^4 promastigote/ml) at a concentration (30 mg/ml), 4-HBA is the third agent in parasitocidal effect that reach to (48×10^4 promastigote/ml) at a concentration (30 mg/ml), SA is the weakest agent in parasitocidal activity that reach to (44×10^4 promastigote/ml) at a concentration (30 mg/ml). It can be concluded that (CA, FA, SA and 4-HBA) possess

acidal effect on the *Leishmania tropica* promastigotes in vitro.

Keywords *Leishmania tropica* promastigotes · Phenolic compounds · Parasitocidal effect · Sodium stibogluconate

Introduction

Leishmaniasis is a disease caused by obligate intracellular, kinetoplastid protozoa of the genus *Leishmania* (Trypanosomatidae) (Roberts 2006). *Leishmania* has two stages in its life cycle amastigote stage in mammalian host and promastigote stage in vector (Osman et al. 2000). Natural transmission may be zoonotic or anthroponotic, and it is usually by the bite of a phlebotomine sand fly species (Killick-Kendrick 1990; Ready 2008). The disease manifest itself in variety of clinical forms, ranging from self-healing cutaneous lesion to the more serious, potentially fatal visceralizing form, and include the metastasize mucocutaneous form and the post kala-azar dermal Leishmaniasis (Thakur 2000). Leishmaniasis is one of the leading causes of morbidity and mortality and it has been found to be a major global health problem (WHO 2007). It is endemic in 88 countries, with an estimated yearly incidence of 1–1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis, three hundred and fifty million people are estimated to be at risk, and there is an overall prevalence of 12 million cases (Desjeux 1996). Cutaneous and mucocutaneous leishmaniasis are more prevalent in Afghanistan, Iraq, Saudi Arabia and some Latin American countries (WHO 2007).

Proven therapies against human leishmaniasis include pentavalent antimonials (sodium stibogluconate and

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meglumine antimonite), amphotericin B, pentamidine, miltefosine and paromomycin (Berman 1996, 1997). These drugs are unsatisfactory because of their limited efficacy, frequent side effects and increasing drug resistance. Development of resistance by the parasites has been reported (Ephros et al. 1997; Lira et al. 1999; Boelaert et al. 2002). A search for a new active compound with potential Leishmanicidal property remains essential for the development of a new antileishmanial therapy. Extracts from medicinal plants are being widely tested for Leishmanicidal activity (Takahashi et al. 2004).

Phenolic compounds are a group of chemical compounds that are widely distributed in nature. They are a chemical family whose members have one or more hydroxyl groups attached directly to an aromatic ring. Fruits, vegetables, spices and aromatic herbs are natural sources of phenolic compounds. There is significant evidence that phenolic compounds have positive effect on the human health with many recently published studies reporting anti-inflammatory, cardio protective activities, anti-allergic, anti-carcinogenic and UV filtering properties, antioxidant as well as anti-microbial properties (Waterman and Mole 1994). Therefore in the present study aims to investigate the effect of some herbal extract such as phenolic compounds on the viability of *Leishmania tropica* promastigotes in vitro.

Materials and methods

Leishmania tropica isolate was obtained from a research unit in the College of Medicine, AL-Nahrain University, Baghdad, Iraq. It was isolated from infected humans. The strain was diagnosed by isoenzyme assay and cultivated on biphasic culture medium at 25 °C. It's composed of two phases (solid phase and liquid phase). This media is used for cultivation and continuation of promastigotes stage of *Leishmania* (Kagan and Norman 1970; Dawson et al. 1969).

RPMI 1640 (Roswell park memorial institute medium)

This media was prepared by adding 10.4 g of powder media in 900 ml of distilled water and 100 ml of preheated fetal calf serum (55 °C for 50 min) then added 1 ml of previously prepared antibiotic solution and the pH was justified to 7.2. Sterilization has been done by Nalgen filter of 0.22 micrometer then distributed 5 ml of media in sterile tube of 10 ml size and incubated in 37 °C for 24 h to avoid the contamination tubes and the sterile tubes put in 4 °C till use (Ranga and Julia 1997).

Growth curve

Promastigotes were cultured in RPMI-1640 with 10 % fetal calf serum medium and incubated in 26 °C. Counting the promastigote each 24 h was performed to determine logarithmic growth phase. The promastigote increased in number in the first, second, third days and reach the peak in the fourth day, the fourth day represent the logarithmic phase (Fig. 1).

Preparation of samples

Assays on L. tropica promastigote were performed as follows Promastigote were cultured in RPMI-1640 medium with 10 % fetal calf serum and incubated in 26 °C, promastigotes in the logarithmic growth phase were then added to each well of a 96-microtiter plate (100 µL). Different concentrations of agent were added on the parasite in each well (50 µL). Further two controls samples were used included: growth medium without agent and growth medium containing studied the anti-Leishmanial drug (Sodium stibogluconate) (20 µg/ml) (Darrell et al. 2002). The plate was incubated in 26 °C for 72 h. Growth was measured in each well through counting the promastigotes after (72 h) by the conventional slide chamber method (Carrio et al. 2001). The parasitocidal effect, the rate of parasitic killing after adding a certain dose of a specific experimental drug, was calculated as the number of dead parasites/total number of parasites (dead + alive) multiplied by 100. This index ranges between a minimum of zero to a maximum of 100. The higher the index the stronger is the effect (Najim et al. 1998).

Preparation of the tested chemical agents

Sodium stibogluconate A 30 ml vial was purchased from (Kolkata, India), patch no: (9p12004). It is containing 100 mg/ml antimony as active ingredient diluted down to 20 µg/ml. This drug was used as a control (Darrell et al. 2002).

The solvent (acetone) A 1,000 ml bottle of acetone solution (100 %) was purchased from (Medex Company, patch no: (200-662-2). The solution of acetone (50 %) prepared by adding acetone: water (50:50 v/v). This solution was used as a solvent to the tested chemical agent (Chong et al. 2009).

Phenolic compounds

- (1) *Caffeic acid* A 10 g crude powder of caffeic acid (CA) was purchased from Sigma Aldrich Company (Batch no. 21909058). Using acetone 50 % (as a

Fig. 1 Line graph showing the mean count of parasites in culture within 5 days after initial inoculation

solvent) and stirrer to prepare five different concentration of CA (10, 15, 20, 25, 30) mg/ml.

- (2) *Ferulic acid* A 5 g crude powder of ferulic acid (FA) was purchased from Sigma Aldrich Company (Batch no. 76755-369). Using acetone 50 % (as a solvent) and stirrer to prepare five different concentration of FA (10, 15, 20, 25, 30) mg/ml.
- (3) *Syringic acid* A 5 g crude powder of syringic acid (SA) is purchased from Sigma Aldrich Company (Batch no. 100989620). Using acetone 50 % (as a solvent) and stirrer to prepare five different concentration of SA (10, 15, 20, 25, 30) mg/ml.
- (4) *4-Hydroxybenzoic acid* A 50 g powder of 4-hydroxybenzoic acid (4-HBA) is purchased from Sigma Aldrich Company (Batch no. 1001034199). Using acetone 50 % (as a solvent) and stirrer to prepare five different concentration of 4-HBA (10, 15, 20, 25, 30) mg/ml.

Result

The effect of increasing concentration of

Phenolic compounds

After adding 10 mg of CA, FA, SA and 4-HBA the parasitidal effect is significantly increased by a mean (24×10^4 , 16×10^4 , 16×10^4 , 13×10^4 promastigote/ml) respec-

tively compared to baseline controls. After each successive increase in concentration the parasitidal effect of CA, FA, SA and 4-HBA is significantly increased to reach a maximum increase by a mean (53×10^4 , 50×10^4 , 44×10^4 , 48×10^4 promastigote/ml) respectively compared to baseline control ($5-6 \times 10^4$ promastigote/ml). The Cohen's d effect size of CA, FA, SA, and 4-HBA is increased from (4.4, 5.7, 6.7, 3.2) respectively after 10 mg concentration to reach a maximum of CA (10.8), FA (9.6), SA (10.5), 4-HBA (5.2) after the highest concentration of 30 mg. The concentration of added CA, FA, SA, and 4-HBA showed a statistically significant ($P < 0.001$) positive strong linear correlation ($r = 0.93$, $r = 0.96$, $r = 0.96$, $r = 0.89$) respectively with parasitidal effect. The regression model explaining the effect of CA, FA, SA, and 4-HBA concentration was statistically significant ($P < 0.001$) and able to explain (87, 93, 93, 78 %) respectively of variation in the dependent variable (parasitidal effect). For each one mg increase in CA, FA, SA, and 4-HBA concentration the parasitidal effect is expected to significantly increase by a mean of (1.69, 1.65, 1.47, 1.66) respectively.

Sodium stibogluconate

After adding 20 μ g/ml of Sodium stibogluconate the parasitidal effect is significantly increased by a mean of 79×10^4 promastigote/ml compared to baseline control (8×10^4 promastigote/ml). The Cohen's d effect size reaches 26.3 after 20 μ g/ml dose (Fig. 2).

Comparing the effect of 5 additives

There was no obvious or statistically significant difference in mean parasitocidal effect at baseline control for the first 4 additives: CA, FA, SA and 4-HBA. The mean parasitocidal effect for 5th experiment baseline control (for sodium stibogluconate) was slightly higher (8×10^4 promastigote/ml) than the remaining 4 experiments ($5\text{--}6 \times 10^4$ promastigote/ml). This difference would not affect the comparison of “changes” in parasitocidal effect after each specific concentration between different additives compared to baseline controls (Fig. 3).

After 10 mg concentration

There was a statistically significant ($P < 0.001$) difference in mean parasitocidal effect after 10 mg concentration between the 4 types of tested additives. The lowest parasitocidal effect was observed with 4-HBA (19×10^4 promastigote/ml). It will therefore be used as the reference (comparison) group. The strongest effect was observed with CA (29×10^4 promastigote/ml), resulting in a Cohen's d effect size of 1.6, which is significantly higher compared to the reference group. Both ferulic and SA showed a slightly higher parasitocidal effect (21×10^4 promastigote/ml), which was not significantly higher than that of the reference group (4-HBA). The Cohen's d effect size for both groups (ferulic and SA) was of small magnitude (0.5) (Fig. 4).

After 15 mg concentration

There was a statistically significant ($P < 0.001$) difference in mean parasitocidal effect after 15 mg concentration between the 4 types of tested additives. The lowest

Fig. 2 Line graph showing the linear trend of mean parasitocidal effect (rate of parasitic killing) with dosage of 5 additives

Fig. 3 Dot diagram with error bars showing the mean (with its 95 % confidence interval) parasitocidal effect (rate of parasitic killing) at baseline (controls) between 5 study groups

Fig. 4 Dot diagram with error bars showing the mean (with its 95 % confidence interval) parasitocidal effect (rate of parasitic killing) after a dosage of 10 mg of additive between 4 study groups

parasiticidal effect was observed with 4-HBA (27×10^4 promastigote/ml) (Fig. 5). It will therefore be used as the reference (comparison) group. The strongest effect was observed with CA (35×10^4 promastigote/ml), resulting in a Cohen's *d* effect size of (1.5), which is significantly higher compared to the reference group. FA showed a slightly higher parasiticidal effect (28×10^4 promastigote/ml), which was not significantly higher than that of the reference group (4-HBA), SA have the same parasiticidal effect of 4-HBA (27×10^4 promastigote/ml). The Cohen's *d* effect size for (FA and SA) was of small magnitude (0.2, 0) respectively (Fig. 6).

After 20 mg concentration

There was a statistically significant ($P < 0.001$) difference in mean parasiticidal effect after 20 mg concentration between the 4 types of tested additives. The lowest parasiticidal effect was observed with SA (35×10^4 promastigote/ml). It will therefore be used as the reference (comparison) group (Fig. 7). The strongest effect was observed with CA (43×10^4 promastigote/ml), resulting in a Cohen's *d* effect size of (1.3), which is significantly higher compared to the reference group. Both Ferulic and 4-HBA showed a slightly higher parasiticidal effect (36×10^4 promastigote/ml), which was not significantly higher than that of the reference group (SA). The Cohen's *d* effect size for both FA (0.3) and for 4-HBA (0.1) (Fig. 8).

After 25 mg concentration

There was not a statistically significant ($P < 0.08$) difference in mean parasiticidal effect after 25 mg dose between the 4 types of tested additives. The lowest parasiticidal effect was observed with SA (43×10^4 promastigote/ml). It will therefore be used as the reference (comparison)

Fig. 5 Dot diagram with error bars showing the mean change (with its 95 % confidence interval) in parasiticidal effect (rate of parasitic killing) after a dosage of 10 mg of additive compared to baseline (control) between 4 study groups

Fig. 6 Dot diagram with error bars showing the mean (with its 95 % confidence interval) parasiticidal effect (rate of parasitic killing) after a dosage of 15 mg of additive between 4 study groups

Fig. 7 Dot diagram with error bars showing the mean change (with its 95 % confidence interval) in parasiticidal effect (rate of parasitic killing) after a dosage of 15 mg of additive compared to baseline (control) between 4 study groups

Fig. 8 Dot diagram with error bars showing the mean (with its 95 % confidence interval) parasiticidal effect (rate of parasitic killing) after a dosage of 20 mg of additive between 4 study groups

Fig. 9 Dot diagram with error bars showing the mean change (with its 95 % confidence interval) in parasitidal effect (rate of parasitic killing) after a dosage of 20 mg of additive compared to baseline (control) between 4 study groups

Fig. 10 Dot diagram with error bars showing the mean (with its 95 % confidence interval) parasitidal effect (rate of parasitic killing) after a dosage of 25 mg of additive between 4 study groups

group (Fig. 9). The strongest effect was observed with CA (49×10^4 promastigote/ml), resulting in a Cohen's d effect size of (1), which is significantly higher compared to the reference group. Both Ferulic and 4-HBA showed a slightly higher parasitidal effect (46×10^4 promastigote/ml), which was not significantly higher than that of the reference group (SA). The Cohen's d effect size for ferulic and 4-HBA was of small magnitude (0.5, 0.3) respectively (Fig. 10).

After 30 mg concentration

There was a statistically significant ($P < 0.003$) difference in mean parasitidal effect after 30 mg dose between the 4 types of tested additives. The lowest parasitidal effect was observed with SA (49×10^4 promastigote/ml) (Fig. 11). It will therefore be used as the reference

Fig. 11 Dot diagram with error bars showing the mean change (with its 95 % confidence interval) in parasitidal effect (rate of parasitic killing) after a dosage of 25 mg of additive compared to baseline (control) between 4 study groups

Fig. 12 Dot diagram with error bars showing the mean (with its 95 % confidence interval) parasitidal effect (rate of parasitic killing) after a dosage of 30 mg of additive between 4 study groups

(comparison) group. The strongest effect was observed with CA (58×10^4 promastigote/ml), resulting in a Cohen's d effect size of (1.5), which is significantly higher compared to the reference group. Both Ferulic and 4-HBA showed a slightly higher parasitidal effect (54×10^4 promastigote/ml), which was not significantly higher than that of the reference group (SA). The Cohen's d effect size for ferulic and 4-HBA was of small magnitude (0.8, 0.5) respectively (Fig. 12).

Discussion

Leishmaniasis are a group of disease caused by Leishmania species, the disease is considered as a major public health problem in 82 countries in the world causing morbidity and mortality (WHO 1990).

There is a general lack of effective and in expensive chemotherapeutic agents for the treatment of leishmaniasis. Although trivalent antimonial like potassium antimonyl

tartrate and pentavalent antimonial drugs are the first-line treatment for this disease, with amphotericin B and pentamidine being used as alternative drugs, all of these have serious side effects and resistance has become a challenging problem. Therefore, new drugs are urgently required, natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans (Wright and Phillipson 1990). In the vast areas of the world, modern drugs are simply not available or if they are available they often prove to be too expensive. The majority of drugs active against infectious agents are in fact derived from natural products (Geoffrey 1996). Different plants of medicinal value are used traditionally worldwide for the treatment of leishmaniasis (Khalid et al. 2005). In the literature there are several reports on the activity of a variety of crude natural extracts, especially from plants collected in tropical zones against *leishmania* species (Fournet and Barrios 1994; Fournet et al. 1996; Akendengue et al. 1999; Weniger et al. 2001; Fournet and Munoz 2002). The parasite cultured in RPMI 1640 medium and counted the number of promastigote daily to determine the logarithmic growth phase, the fourth day (96 h) represent the logarithmic growth phase and the numbers reach to (4×10^6) promastigote/ml (Fig. 13).

The study of Mobarak (2008) shows the number of CL growing in RPMI 1640 medium reach to (2.8×10^6) promastigote/ml in logarithmic growth phase. The study of Ibrahim et al. demonstrate the number of CL and VL growing in RPMI 1,640 medium reach to (5×10^7) promastigote/ml in logarithmic growth phase (Ibrahim et al. 1994). Another study by Steiger and Steiger, demonstrate the number of *Leishmania donovani* and *Leishmania braziliensis* reach to (4×10^7) promastigote/ml in logarithmic growth phase (Steiger and Steiger 1977).

Fig. 13 Dot diagram with error bars showing the mean change (with its 95 % confidence interval) in parasiticidal effect (rate of parasitic killing) after a dosage of 30 mg of additive compared to baseline (control) between 4 study groups

The effect of phenolic compounds on *Leishmania* parasite viability is not yet known, and, to date, no other outcomes studies have been announced.

It is observed that the four tested agents (CA, FA, SA and 4-HBA) have different parasiticidal effect depending on the difference in concentration after duration 72 h. The parasiticidal effect of each agent is tested according to five different concentrations (10, 15, 20, 25, 30 mg/ml).

This study demonstrates that CA has a strong parasiticidal activity depending on the variation in concentration after duration of 72 h. This study showed the mean of parasiticidal effect of CA at 10 mg/ml is (24×10^4) promastigote/ml as compared to baseline control. After each successive increase in concentration the parasiticidal effect is increased to reach a mean (30×10^4) promastigote/ml in a concentration 15 mg/ml. The parasiticidal effect more increased when adding (20, 25, 30 mg/ml) to reach a mean $(38 \times 10^4, 44 \times 10^4, 53 \times 10^4)$ promastigote/ml respectively. These observations are in consistency with the study of Chong et al., reported that CA inhibits the radial growth of *Ganoderma Boninense* (Chong et al. 2009). Another study by Ismaiel and Pierson investigated the effect of CA against *Clostridium botulinum*, they state that CA reduces the viability of *Clostridium* spores (Ismaiel and Pierson 1990). The antimicrobial effect of CA may be attributed to the interaction with metal ions such as iron which is essential for microbial growth (Kontoghiorghe et al. 1986). Furthermore CA could cause change in the structure of cytoplasmic proteins that inhibit cell division (Cowan 1999). In another report, CA was also found inhibiting the growth of sweet potato pathogenic fungi, inhibitory activity reported and suggests high periderm CA levels contribute to the storage root defense chemistry of some sweet potato genotypes (Harrison et al. 2003).

In this study the effect of FA on the viability of *Leishmania tropica* promastigote are investigated and showed a significant impact on their number, an increase in FA concentration has a major effect on *L. tropica* promastigotes viability. This study demonstrated that the parasiticidal effect of FA at (10 mg/ml) is (16×10^4) promastigote/ml compared to baseline control and the parasiticidal rate increased after increasing in concentration (15, 20, 25, 30 mg/ml) to reach a mean $(23 \times 10^4, 31 \times 10^4, 42 \times 10^4, 50 \times 10^4)$ promastigote/ml respectively. In this study FA is considered the best parasiticidal agent after CA in comparison to the other tested agents. This study is consistency with the study of Felaih et al. (2011), demonstrated in vitro study that phenolic compounds (CA and FA) have a fatal effect on the viability of protozoans under different concentration and different durations (Felaih et al. 2011). The study of Aziz, which states that FA is able to inhibit growth of *seed-born* fungi, the possible explanation of the antimicrobial effect of FA is

the ability of FA to interrupt the activity of the enzyme that responsible for DNA replication such as topoisomerase (Aziz et al. 1998). Another study by Franke et al., states that FA inhibits the respiratory syncycial virus reproduction (Natalia 2013). The study of Gardjeva et al. (2007), reported that FA has bactericidal activity against streptococcus beta-haemolyticus.

In this study showed the parasitocidal effect of SA at (10 mg/ml) is (16×10^4 promastigote/ml) compared to baseline control. The parasitocidal effect increased after increase in concentration to reach in a mean (22×10^4 promastigote/ml) at a concentration (15 mg/ml) and in a concentration (20, 25, 30 mg/ml) the parasitocidal effect reach to a mean (30×10^4 , 38×10^4 , 44×10^4 promastigote/ml) respectively. This study demonstrates that SA has a weak parasitocidal activity in comparison to the other tested agents.

This study is consistence with the experiment conducted by Chong et al. (2009), SA was found to be very fungi toxic to *Ganoderma boninense*, when the concentration was increased the pathogen was fully inhibited. Many works on plants have demonstrated the fungi toxicity effect of SA, in resistance raspberry to fungus *Didymella*, SA was found accumulated in the bordering zone of lesion forming a barrier to the fungus (Kozłowska and Krzywanski 1994). In sugar cane plant, which is highly resistant to smut disease showed a major accumulation pattern of SA when interact with the pathogen (De Armas et al. 2007).

In this study the effect of 4-HBA on the viability of *Leishmania tropica* promastigotes were investigated an increase in 4-HBA concentration has a major effect on *Leishmania tropica* promastigotes viability. This study demonstrated the mean at (10 mg/ml) is (13×10^4 promastigote/ml) as compared to baseline control. The parasitocidal effect increased as the concentration is varying (15, 20, 25, 30 mg/ml) to reach a mean (22×10^4 , 31×10^4 , 41×10^4 , 48×10^4 promastigote/ml) respectively. In this study 4-HBA has a best paracitocidal activity after CA and FA in a comparison to other tested chemical agents. Cho et al. (1998), showed the role of 4-HBA in rice hull that have antimicrobial effect against various microorganisms, most of the gram-positive and some gram-negative bacteria. A study by Chong et al., reported that the synergy effect of SA, CA and 4-HBA against *Ganoderma boninense*, either syringic with 4-HBA or caffeic with 4-HBA or syringic with CA or the combination of all the three phenolics totally inhibited the growth of *Ganoderma Boninense*, the pathogen failed to grow in a combination of a high concentration of all the three phenolics but the combination of lower concentration of the phenolics which poorly inhibited the growth of the pathogen, but a better performance were shown in a combination of CA and 4-HBA in a high concentration (Chong et al. 2009).

Maddox et al. (2010), observed that phenolic compounds that have antibacterial activity against *Xylella fastidiosa*.

Winkelhausen et al. (2005), demonstrated that the phenolic compounds derived from olive pomace inhibited the growth of fungi (*Alternaria solani*, *Botrytis cinerea* and *Fusarium culmorum*). Different researchers have also reported that phenolic compounds from different plant sources could inhibit various pathogens (Nychas 1995; Smid and Gorris 1999; Prashanth et al. 2001; Kim et al. 2005). The antimicrobial activities of phenolic compounds may involve multiple modes of action. For example, essential oils degrade the cell wall, interact with the composition and disrupt cytoplasmic membrane (Sikkema et al. 1994; Helander et al. 1998; Ultee et al. 1999; Lambert et al. 2001), damage membrane protein, interfere with membrane integrated enzymes (Raccach 1984), cause leakage of cellular components, coagulate cytoplasm, deplete the proton motive force, change fatty acid and phospholipid constituents, impair enzymatic mechanisms for energy production and metabolism, alter nutrient uptake and electron transport (Taniguchi et al. 1988), influence the synthesis of DNA and RNA and destroy protein translocation and the function of the mitochondrion in eukaryotes (Nychas 1995; Raccach 1984). All of these mechanisms are not separate targets; some are affected as a consequence of another mechanism being targeted. The mode of action of antimicrobial agents also depends on the type of microorganisms and is mainly related to their cell wall structure and the outer membrane arrangement (Kalemba and Kunicka 2003; Burt 2004).

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