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MICROTOX TEST AS A TOOL TO ASSESS ANTIMICROBIAL PROPERTIES OF HERBAL INFUSIONS USED IN URINARY TRACT INFECTIONS

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Abstract: Herbs can be used preventatively, to aid treatment and to ease the symptoms of urinary tract infections (UTIs). Traditional medicine uses herbal infusions such as: lingonberry leaf (*Vitis idaeae folium*), birch leaf (*Betulae folium*) wild thyme extract (*Serpylli herba*), dwarf everlast flower (*Helichrysi inflorescentia*), goldenrod (*Virgaureae herba*, also called *Solidaginis herba*), restharrow root (*Ononidis radix*), agrimony (*Agrimoniae herba*), rowanberry (*Sorbi aucupariae fructus*), black elderberry (*Sambuci fructus*), and juniper berry (*Juniperi fructus*). This study examined which of the herbal infusions used in treatment and reduction of symptoms of UTIs have the greatest efficacy, and at which concentration levels (5.85 mg/mL; 0.59 mg/mL; 0.29 mg/mL). The results obtained suggest that the Microtox test can be successfully used to assess the healing properties of herbal infusions. The results of the experiments carried out using the Microtox test show that both in preventative medicine, as well as in aiding treatment of UTIs, the biggest benefit is brought by herbal infusions of wild thyme extract and birch leaf (at all concentrations), and also by infusions at higher concentrations (approx. 5.85 mg/mL) of agrimony, dwarf everlast flower, lingonberry leaf, artichoke herb, goldenrod, and juniper berry.

Keywords: herbs, herbal infusions, Microtox, urinary tract infection, *Virbio fischeri*

The Microtox test is one of the first commercially available biotests used to measure the acute toxicity of environmental samples (surface and underground water, sea water and water intended for consumption, sewage, sediments, soil) (1, 2). The test appeared on the market in the 70's (3). The developers of this test did not suggest that the test be used to measure the toxicity of herbs. Currently there are not many examples of the Microtox test being used to measure the toxicity of medicinal plants (4). In one publication the Microtox test was used to assess the toxicity of common thyme (*Thymus vulgaris* L.), medicinal sage (*Salvia officinalis* L.) and the stinging nettle (*Urtica dioica* L.), but in the context of assessing environmental pollution (5), and not in order to assess the natural toxicity of the compounds produced by the plants.

Plants have been used by humans to treat illnesses since the dawn of time. Modern herbal medicine is seen as an essential part of traditional medicine (6), and herbs are used preventively, as part of the course of treatment, and also to ease the symptoms, in many illnesses (7).

To treat urinary tract infections (UTIs), the following herbs (amongst others) are used: birch leaf (*Betulae folium*), juniper berry (*Juniper fructus*), black elderberry (*Sambuci fructus*), lingonberry leaf (*Vitis idaeae folium*), and wild thyme extract (*Serpylli herba*) (8).

UTIs are considered the most frequently occurring bacterial infection in humans (9). 75%-95% of infections are caused by the *Escherichia coli* bacteria (10). Statistics show that around 40%-50% of women have experienced a UTIs at least once in their lives. In 25%-30% of cases the disease returns within 6-12 months of the initial infection (11, 12). Because of female anatomy (i.e., close proximity of the urethra to the anus), women have a significantly higher chance of contracting a UTIs than men, whose rate of infection is only 12% (11). A higher risk is of infection is found in babies and children (13, 14), pregnant women (15), elderly people (14), patients with spinal cord injuries and/or with catheters (9, 16), patients suffering from diabetes (17), multiple sclerosis (18), immunodeficiency (19), and urological diseases (9).

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The recent spread of interest in the usage of herbal medicine related to UTI treatment is due to the increasing resistance of *Escherichia coli* to antibiotics (12). *Escherichia coli* bacteria are very similar to *Vibrio fischeri*, which are used in the Microtox test. This resemblance includes both their structure (rod-shaped, ciliated, Gram-negative), along with their ability to create a biofilm (20). Also, the genes responsible for bioluminescence in *Vibrio fischeri* bacteria can be cloned to the cells of *Escherichia coli* (21, 22). These similarities have encouraged the authors of this study to consider the possibility of using the Microtox test to assess herbal infusions. The specific measurement of the healing potential would be the toxicity of the infusions, as assessed using the Microtox test.

EXPERIMENTAL

Materials

Luminescent bacteria *Vibrio fischeri*, reconstitution solution, diluent (Strategic Diagnostic, 2% NaCl solution) and Osmotic Adjusting Solution (OAS, 22% NaCl) from the AZUR Environmental company. Spring mineral water ('Żywiec' brand, Poland) of mineral content 230 mg/L was used to create the herbal infusions together with the following herbs: a mixture of herbs known to be beneficial for healthy kidney function: oat herb (*Avena sativa herba*), artichoke herb (*Cynarae scolymus herba*) (from the Dary Natury company); lingonberry leaf (*Vitis idaeae folium*), birch leaf (*Betulae folium*) (from the herbal company 'Kawon-Hurt' Nowak Sp.J.); wild thyme extract (*Serpylli herba*), heather flowers (*Callunae vulgaris flos*), dwarf everlast flowers (*Helichrysi inflorescentia*), goldenrod (*Virgaureae herba*, also called *Solidaginis herba*), equisetum stem (*Equiseti herba*), restharrow root (*Ononidis radix*), agrimony (*Agrimoniae herba*), rowanberry (*Sorbi aucupariae fructus*) black elderberry (*Sambuci fructus*), juniper berry (*Juniperi fructus*) (from the Flos company).

Preparing the herbal infusions

The herbal infusions were created by pouring 200 mL of boiling Żywiec mineral water (mineral content 230 mg/L) on each herbal sample in turn. The herbs were covered and left to steep for 15 min. The infusion was left to cool down, and then the reaction was measured using a pH meter (Hanna HI 221). The pH meter was calibrated each day using a pH buffer solution (Merck) of pH 4.0 and 9.0. The acute toxicity of the infusions was then measured, using the Microtox test.

The herbal solutions at concentrations of 5.85 mg/mL were prepared by weighing and adding approximately one teaspoon, i.e., 1.17 g of each herb (weighed using Radwag AS 160.3Y scales). The infusions at concentrations of 0.59 mg/mL were prepared from: rowanberry (0.1314 g), agrimony herbs (0.1172 g), black elderberry (0.1182 g), wild thyme (0.1173 g).

The herbal solutions at concentrations of 0.29 mg/mL, were prepared from: oat herbs (0.0586 g), birch leaf (0.0592 g), everlast flowers (0.0589 g), lingonberry leaf (0.0597 g), restharrow root (0.0585 g), goldenrod (0.0586 g), artichoke (0.0587 g), equisetum stem (0.0581g), juniper berry (0.0630 g),

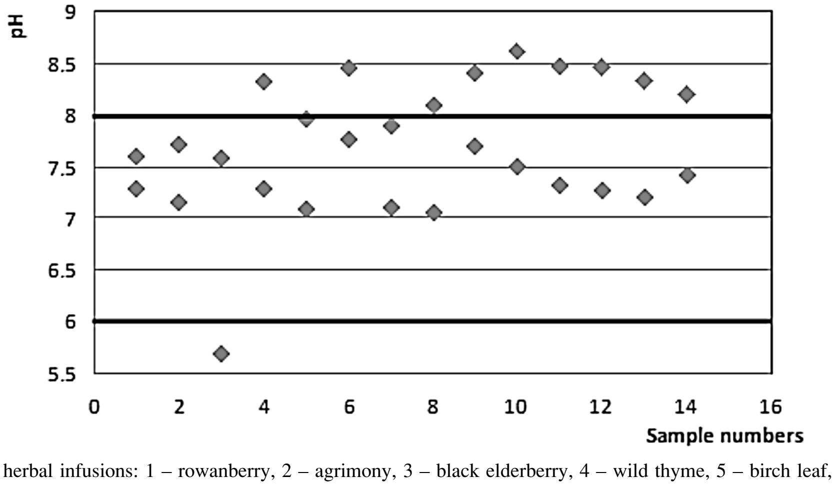

Figure 1. pH of herbal infusions: 1 – rowanberry, 2 – agrimony, 3 – black elderberry, 4 – wild thyme, 5 – birch leaf, 6 – oat herb, 7 – heather, 8 – dwarf everlast flower, 9 – lingonberry leaf, 10 – restharrow root, 11 – equisetum stem, 12 – goldenrod, 13 – artichoke, 14 – juniper berry

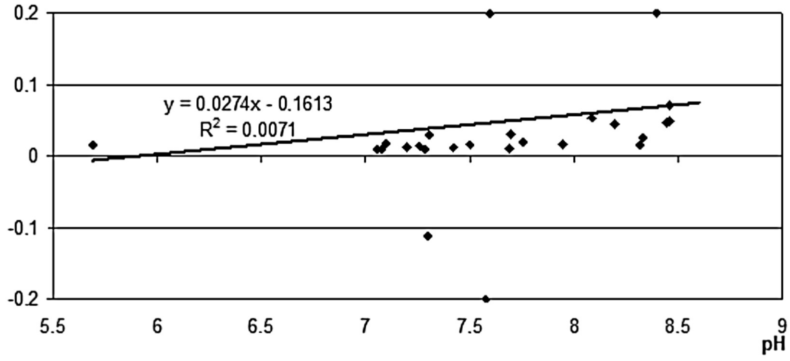

Figure 2. Correlation between inverse toxicity of herbal infusion (1/toxicity) and pH

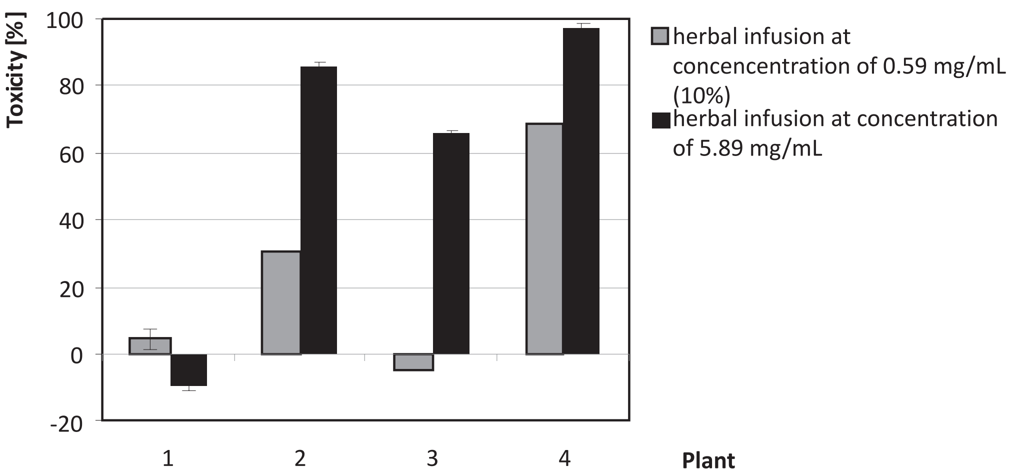

Figure 3. Toxicity of herbal infusions at concentrations of 0.59 mg/mL, and 5.85 mg/mL: 1 – rowanberry, 2 – agrimony, 3 – black elderberry, 4 – wild thyme

heather (0.0594 g), rowanberry (0.1314 g), agrimony (0.1172 g), black elderberry (0.1182 g), wild thyme extract (0.1173 g).

Toxicity analysis using the Microtox test

The toxicity of the samples was measured using the Microtox test, and the AZUR Environmental 500 analyser. The measurements were taken 3 times, and the average toxicity was calculated from those three readings. In the test a selective strain of luminescent marine bacteria *Vibrio fischeri* was used. The toxicity of the samples was measured according to the company's standard procedure requirements (SDI). One mL of the sample and 1 mL of the diluting agent (2% NaCl solution, AZUR Environmental). 200µL of OAS (Osmotic Adjusting Solution, 22% NaCl, AZUR Environmental), were also added to the samples, to ensure the correct osmotic pressure, along with 100 µL of *Vibrio fischeri* bacteria, suspended in reconstitution solution (AZUR Environmental). The toxicity was checked after 30 min of incubation at temperatures of +15°C ± 0.5°C. The sample should show a toxicity of between pH 6.0-8.0 on testing. The results were calculated using the company software, MicrotoxOmni (AZUR Environmental).

RESULTS AND DISCUSSION

The level of toxicity in the Microtox test can be measured from samples whose pH is between 6.0-8.0. Therefore, the reaction of each herbal infusion was noted (Fig. 1).

Approximately 35% of the pH readings were not within the range required to show on the Microtox test, i.e., 6.0-8.0. However, our earlier studies have shown that pH can have an effect on the Microtox test at pH < 5.0 and > 8.5 [study under

Table 1. Ingredients, function, and application of the selected herbs used preventatively for UTIs (8).

Name of substance	Main components	Function	Usage
Birch leaf (*Betulae folium*)	Flavonoids, catechin tannins, triterpenes, polyprenols,essential oil, resins, organic acids	Diuretic, bactericidal	Chronic urinary tract diseases, urolithiasis/urinary stones, urinary tract infections
Goldenrod (*Virgaureae herba*, syn. *Solidaginis herba*)	Flavonoids, triterpenoid saponins, diterpene compounds, tannins, essential oil, phenol glycosides, polysaccharides, bitters, mucilaginous compounds, mineral salts	Diuretic, bactericidal, anti-inflammatory	Urinary system inflammation and infection, urinary stones
Oat herb (*Avena sativa herba*)	Mineral compounds, flavonoids, steroidal saponins, sugars, nicotinamide, water soluble silica, macro- and micro-elements	Diuretic	Kidney disease, urinary stones
Restharrow root (*Ononidis radix*)	Flavonoids, triterpenes, tannins, essential oil, organic acids, resins, mineral salts	Anti-inflammatory	Kidney and urinary tract inflammation, urinary stones
Equisetum stem (*Equiseti herba*)	Flavonoids, saponins, mineral salts, organic acids, silicon compounds (20% as water soluble silica), phytosterols, vitamin C, carotenoids, tannins, resin compounds	Diuretic, antibacterial, anti-inflammatory, blood vessel healing	Kidney inflammation, ureteritis, bladder and urethral inflammation, oliguria/hypouresis, urinary stones
Juniper berry *(Juniperi fructus*)	Essential oils (terpenes), flavonoids, tannins, resins, sugars, organic acids, mineral compounds, vitamin C	Diuretic, antibacterial, disinfectants	Urinary tract inflammation, urinary stones
Artichoke herb (*Cynarae scolymus herba*)	Cynarine, caffeic acid and chlorogenic acid, cynaropicrin, triterpenes, flavonoids, sterols, tannins, pectins, vitamins A and B, mineral salts	Diuretic	Kidney diseases, urinary stones
Lingonberry leaf (*Vitis idaeae folium*)	Phenol glycosides (arbutin, 6-acetyl-arbutin), hydroquinone, catechin tannins, flavonoids, ursolic acid, organic acids, mineral salts	Diuretic, bactericidal, anti-inflammatory	Catarrh and inflammation of the urinary tract, urinary stones
Thyme extract *Serpylli herba*)	Essential oils, tannins, flavonoids, bitter compounds, saponins, organic acids, mineral salts	Anti-inflammatory, bactericidal	Urinary tract infections
Heather (flower) (*Callunae vulgaris flos*)	Polyphenolic compounds (flavonoids, quercetyn derivatives, myricetins), procyanidins, tannins, phenol glycosides, phenolic acids, mineral compounds	Diuretic, anti-inflammatory, antibacterial	Diseases and ailments of the kidneys and urinary tract, urinary stones

Table 1. Cont.

Name of substance	Main components	Function	Usage
Dwarf everlast flower (*Helichrysi inflorescentia*)	Flavonoids (isosalipurposides), kaempferol glycosides and apigenins, phthalide compounds, essential oils, tannins, carotenoids, organic acids	Diuretic, bactericidal	Urinary tract infections
Agrimony (*Agrimoniae herba*)	Tannins, ellagic acid derivatives and catechin acid derivatives, flavonoids, quercetyn derivatives, triterpenes,organic acids, bitters, vitamins B_3, C, K, PP, choline, phytosterols, leucocyanidines, mineral compounds	Disinfectant, anti-inflammatory, diuretic	Cystitis
Rowanberry (*Sorbi aucupariae fructus*) bitter	Organic acids (parasorbic, malic), carotenoids, compounds, carbohydrate compounds (D-sorbitol), tannins, vitamins C, B, E, PP and pro-vitamin A, anthocyanins, pectins, tannins, mineral salts	Diuretic	Impaired kidney function
Black elderberry (*Sambuci fructus*)	Anthocyanin glycosides, tannins, Flavonoids, pectins, sugars, B-group vitamins, vitamins C and P, pro-vitamin organic acids, mineral salts	Diuretic, anti-inflammatory	Urinary tract inflammation

review]. Figure 2 shows the correlation between inverse toxicity (1/toxicity on the y axis) and pH of the sample.

Both the slope (gradient) of the straight line and the coefficient R^2 indicate a lack of linear dependence between 1/toxicity and pH. The results above show that the natural chemical compounds produced by the plants most likely have an effect on the toxicity of the infusions (Table 1).

Toxicity measurements were also recorded for the infusions of the aforementioned individual herbs, with a mass of 10% at concentrations of 0.59 mg/mL (Fig. 3) as well as 5% at concentrations of 0.29 mg/mL (Fig. 4).

The obtained results indicate a significant difference between the toxicity measurements recorded from herbal infusions at concentrations of 5.85 mg/mL, and those at 0.59 mg/mL (Fig. 3). From these four herbs (at concentrations of 5.85 mg/mL) only two indicate high toxicity (> 80%): agrimony and wild thyme. In the case of samples at concentrations of 0.59 mg/mL only wild thyme indicates toxicity at a level of approx. 70%. The remaining herbs at a concentration of 0.59 mg/mL are not toxic toward the *Vibrio fischeri* bacteria.

All of the obtained extracts were clear, from transparent to a yellowish hue. The color was not an impediment to assessing the toxicity. The only exception was the infusion from black elderberries at a concentration of 5.85 mg/mL, which was very dark. In the case of this infusion, it is possible that the 60% drop in luminescence is partly caused by the color of the infusion.

The difference between the obtained toxicity of the selected herbal infusions at concentrations of 0.29 mg/mL and 5.85 mg/mL (Fig. 4) is bigger than in the case of the difference in toxicity between solutions at a concentration of 0.59 mg/mL (10%) and 5.85 mg/mL (100%) (Fig. 3). Among ten herbs at concentrations of 5.85 mg/mL, four exhibited a high toxicity (> 80%): birch leaf, dwarf everlast flower, lingonberry leaf, artichoke herb; three showed a medium toxicity, in the range of 60%-80%: restharrow root, goldenrod, juniper berry; whereas the remaining herbal infusions were characterised by low toxicity. The range of toxicity at concentrations of 5.85 mg/mL (after including the results from Fig. 3), can be presented as follows:

dwarf everlast flower = lingonberry leaf > birch leaf = wild thyme > artichoke herb > agrimony > juniper berry > goldenrod > restharrow root > black elderberry > heather > oat herb > equisetum stem > rowanberry

whereas at a lower concentration of 0.29 mg/mL:

birch leaf > artichoke herb > juniper berry = oat herb > goldenrod > dwarf everlast flower > equisetum stem > lingonberry leaf > heather > restharrow root

Among the infusions of various herbs at concentrations of 5.85 mg/mL and 0.29 mg/mL the smallest differences in toxicity were found on observing wild thyme extract and birch leaf. This may indicate that these herbs play a significant role in preventative medicine, as in aiding the treatment of UTIs. The following herbs, which showed a high toxicity (> 80%) in infusions at concentrations of 5.85 mg/mL, may also have a large impact on the preventative care of the urinary tract: agrimony, wild thyme extract, birch leaf, dwarf everlast flower, lingonberry leaf, artichoke herb. Goldenrod and juniper berry also show close to 80% toxicity.

In an infusion of mixed herbs for improved kidney function at a concentration of 5.85 mg/mL the toxicity reached 93%. This mixture included 10% of rowanberries, agrimony, black elderberries and wild thyme extract, along with around 5% of oat herb, birch leaf, dwarf everlast flowers, lingonberry leaf, restharrow root, goldenrod, artichoke herb, equisetum stem, juniper berries, heather. The high toxicity of the infusion of mixed herbs for kidney function is most likely due to the interaction of the various herbs in the mixture.

None of the various herbal infusions at concentrations of 0.59 mg/mL or 0.29 mg/mL indicated a toxicity of 93%, as in the case of the herbal mixture. Taking into account the results of the tests, one can assume that wild thyme extract and birch leaf had the greatest influence on toxicity of the herbal mixture.

CONCLUSION

The obtained results suggest the Microtox test can be used to assess the toxicity of herbal infusions. The results of the tests indicate that wild thyme extract and birch leaf are the most effective herbs for preventative medicine and in aiding treatment of UTIs. The remaining herbs in the kidney function mixture may show additive toxicity, which means that the herbal infusions will not express toxicity individually, but the mixture thereof will be toxic. The tests carried out as described above suggest that the concentration of the herbal infusion also has an effect on its toxicity. Herbs showing a high toxicity (> 80%) in herbal infusions at concentrations of 5.85 mg/mL (agrimony, wild thyme extract, birch leaf, dwarf everlast flower, lingonberry leaf, artichoke herb) do not exhibit high toxicity in infusions at lower concentrations of 0.59 mg/mL or 0.29 mg/mL. The exceptions were wild thyme and birch leaf.

The Microtox test showed that the greatest benefit is to be had from herbal infusions of wild thyme and birch leaf, both in preventative medicine and also in aiding treatment, as well as by the fol-

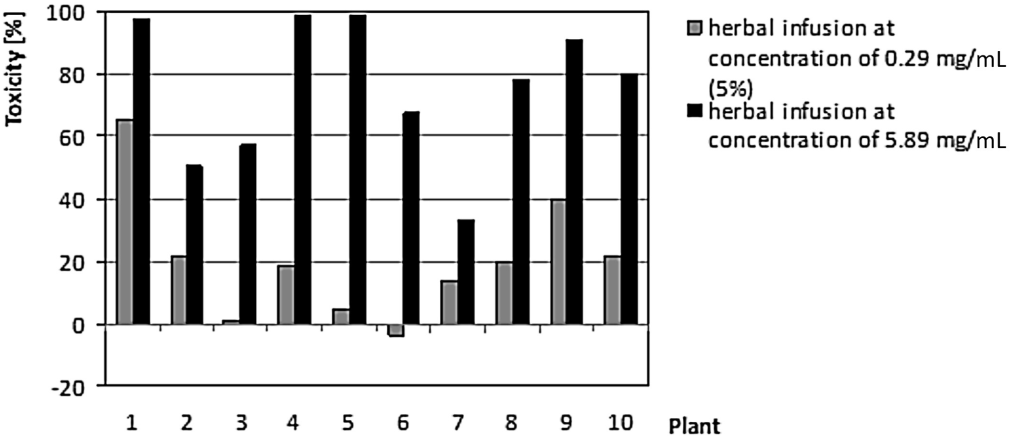

Figure 4. Toxicity of herbal infusions at concentrations of 0.29 mg/mL and 5.85 mg/mL: 1 – birch leaf, 2 – oat herb, 3 – heather, 4 – dwarf everlast flower, 5 – lingonberry leaf, 6 – restharrow root, 7 – equisetum stem, 8 – goldenrod, 9 – artichoke herb, 10 – juniper berry

lowing herbs at higher concentrations of around 5.85 mg/mL: agrimony, dwarf everlast flower, lingonberry leaf, artichoke herb, goldenrod and juniper berry.

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The authors declare that they have no competing interests.

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