

Original Article

SYNERGISTIC EFFECT OF ANTI-OXIDANT, ANTI-TYROSINASE AND ANTI-BACTERIAL ACTIVITIES OF *TRIDAX PROCUMBENS*, *LANTANA CAMARA*, *EUPHORBIA HIRTA* AND *THEVETIA PERUVIANA* PLANT EXTRACTS FOR COSMETIC AND PERSONAL CARE APPLICATIONS

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ABSTRACT

Objective: Herbal skin care products have become increasingly popular in the cosmeceutical industry. Skin care products are used to lighten the skin, improve the skin tone, treat pigmentation disorders and to combat the skin microflora. The approaches in development of skin care products have broadened widely in the recent years. One of the approaches is the utilization of a consortium of herbal extracts that have multiple benefits such as antimicrobial, anti-tyrosinase and antioxidant activities for their combined effects in skin care formulations. The present study is an effort to investigate the anti-tyrosinase, anti-oxidant and antibacterial properties in selected Malaysian plants viz. *Tridax procumbens*, *Lantana camara*, *Euphorbia hirta* and *Thevetia peruviana* towards their application in skincare and personal care formulation. The major objectives of the study are: to extract the bio-active principles from these plants with three different solvents, screen the extracts for anti-tyrosinase activity, anti-oxidant and anti-bacterial activities, evaluate the synergistic effect of polyherbal mix for the development a skincare formulation that has a combined effect of anti-tyrosinase, anti-oxidant and anti-bacterial activities.

Methods: The crude extracts were screened for (i) anti-tyrosinase property by dopachrome method; (ii) anti-oxidant property by DPPH and FRAP assays and (iii) antimicrobial property using disc diffusion and broth dilution methods. Synergistic combination of specific plant extracts rich in anti-tyrosinase, antioxidant and antimicrobial properties were incorporated and evaluated for their benefits in the skincare cream. The skincare cream was evaluated for preservative efficacy by challenging it with test microorganisms: *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* as described in British Pharmacopoeia 2002.

Results: The anti-oxidant activity of plant extracts were evaluated using DPPH and FRAP assays which were found to be correlated. Antioxidant activity was observed to be the highest in methanol extract of *Euphorbia hirta* and acetone extract of *Euphorbia hirta*. The polyherbal extract combination acted as the only preservative in the formulation and recorded 99% mortality of the loaded microorganisms in 7 days. Hence, the polyherbal extract combination would serve as natural preservative augmented with skin lightening benefits replacing the synthetic anti-tyrosinase, anti-oxidant and anti-bacterial compounds. Anti-tyrosinase activity has been reported in *Tridax procumbens*, *Euphorbia hirta* and *Thevetia peruviana* for the first time in this study.

Conclusion: Remarkable anti-bacterial activity was observed in acetone extracts of *Tridax procumbens*. Synergistic effect of the polyherbal mix was confirmed for anti-oxidant activity and antibacterial activity. However, synergistic effect of the polyherbal mix for anti-tyrosinase activity was not significant in comparison to the individual activity of the selected plants.

Keywords: Medicinal plants, Anti-tyrosinase, Anti-oxidant, Antimicrobial, Polyherbal mix, synergy, Natural preservative.

INTRODUCTION

The natural substitutes for synthetic chemicals are most sought in demand globally and driven by growing consumer awareness. In the recent past, the need to explore the use of herbal plants has plunged to greater heights. Over 35,000 plants have been reported for medicinal benefits and they have been in use by human beings [1]. Herbal consumer products are formed as a result of extracting the beneficial components (bio-active principles) from plants using specific solvents of appropriate polarity. Hence, the selection of solvents plays a major role in extraction of anti-tyrosinase, anti-oxidant and anti-bacterial compounds. Maceration is one of the most widely used solvent extraction methods because it minimizes the degradation of thermolabile compounds [2]. In this study, maceration method is employed to extract the bio-active principles from the four selected plants.

The anti-oxidant, anti-tyrosinase and anti-bacterial activities have many applications in cosmetic and personal care industry to reduce hyperpigmentation [3], reduce aging process, protect skin against oxidative damage [4,5], hair growth promotion [6] and in medical industry as bio-pesticide, reduce virulence of melanized microbes in host organism, antibiotics, etc [7,8].

The purpose of evaluating the synergistic effect is to achieve higher beneficial outcome that can surpass the results of each individual plant sample. Pharmacological uses of *Tridax procumbens*, *Lantana camara*, *Euphorbia hirta* and *Thevetia peruviana* have been reported [9-12]. However, the synergistic effect of these plants has not been identified yet. Hence, the present study represents the most comprehensive method on the synergistic effect of these combined plant extracts for anti-oxidant, anti-tyrosinase and anti-bacterial activities. The objectives of this study were to (1) evaluate and compare anti-oxidant property by DPPH and FRAP assay; (2) evaluate anti-tyrosinase activity; (3) evaluate anti-bacterial activity and (4) evaluate synergistic effect for anti-oxidant, anti-tyrosinase and anti-bacterial activities in *Tridax procumbens*, *Lantana camara*, *Euphorbia hirta* and *Thevetia peruviana*.

MATERIALS AND METHODS

Materials

1, 1-diphenyl-2-picrylhydrazyl (DPPH) and mushroom tyrosinase enzyme were purchased from Sigma-Aldrich (M) Sdn Bhd. Solvents such as petroleum ether, acetone and methanol, Butylated hydroxytoluene (BHT) and L-3, 4-Dihydroxyphenylalanine (L-DOPA)

were obtained from R & M Chemicals. Susceptibility test discs were received from Becton Dickinson.

Kojic acid and Muller Hinton (MH) Agar and Luria Agar media were purchased from Himedia. Fresh leaves of *Tridax procumbens*, *Lantana camara*, *Thevetia peruviana* and aerial parts of *Euphorbia hirta* were collected from Sungai petani in November 2010.

Sample preparation

Collected leaves of *Tridax procumbens*, *Lantana camara*, *Thevetia peruviana* and aerial parts of *Euphorbia hirta* were shade dried for 3 weeks and coarsely powdered. The powdered plant samples were macerated with solvents such as petroleum ether, acetone and methanol in sequence from non-polar solvent to polar solvent in a ratio of 1:6. The supernatant was removed from the retentate using Whatman filter paper No. 1 and concentrated using a rotary evaporator at a temperature less than the boiling point of the solvent.

Determination of antioxidant activity

The antioxidant activity of samples was experimentally measured by DPPH assay and FRAP assay which works on DPPH radical scavenging and ferrous reducing properties respectively.

DPPH assay

The amount of DPPH radical scavenging activity was determined as described by Kamkar et al., [13]. The diluted working solutions of the test extracts were prepared with respective solvents. BHT was used as standard. Experiment involved mixing of 50 µl of test extracts with 5 ml of 0.004% DPPH solution. The mixed solution was incubated for 30 minutes in dark and optical density was measured at 517 nm using a spectrophotometer. The optical density was recorded and % DPPH scavenging was calculated. Higher the scavenging effect, lower is the optical density of the mixed solution.

FRAP assay

Ferrous reducing antioxidant power of the plant extracts were determined according to Benzie and Strain [14]. In the presence of antioxidant, the ferrous present in FRAP reagent gets reduced to ferric form. Ferrous sulphate is used as standard. Stock solution was prepared consisting of (i) 300 mM acetate buffer (3.1 g of C₂H₃NaO₂·3H₂O and 16 ml of C₂H₄O₂), (ii) 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl and (iii) FeCl₃·6H₂O solution in the ratio 10:1:1. Individual plant extracts (100 µl) were allowed to react with 3 ml of freshly prepared FRAP solution. The reaction mixture was incubated at 37 °C for 4 minutes and the optical density of the reaction mixture was measured at 593 nm before incubation and after incubation. FRAP value of the sample was calculated.

Determination of anti-tyrosinase activity

The mushroom tyrosinase inhibition method as stated by Narisa et al. [15] with DOPA as substrate was used to determine the anti-tyrosinase activity of plant extracts with slight modifications. Stock solution of plant extracts at 2, 0.2, 0.02, 0.002 and 0.0002 mg/ml concentration were prepared. The microtitre plate was filled with 140 µl of Phosphate buffer (pH 6.8), 20 µl of 1000 u/ml tyrosinase, 20 µl of plant extract and 20 µl of 0.85 mM L-DOPA. Kojic acid was used as the positive control. The negative control was prepared by replacing tyrosinase with phosphate buffer. The microplate was incubated for 10 minutes at room temperature and absorbance was recorded at 475 nm using a microplate reader. These absorbance values correspond to the amount of dopachrome produced.

Anti-bacterial activity of the plant extracts was determined using Disc diffusion test (Qualitative test) and Micro Broth Dilution Test (Quantitative).

Disc diffusion test

Antibacterial activity was initially screened using disc diffusion method. This method [16] reveals the sensitivity or resistance of bacteria to the plant extracts based on the zone of inhibition. Overnight broth cultures of Gram-positive bacteria - *Micrococcus luteus* and Gram-negative bacteria - *Pseudomonas aeruginosa* were

prepared. Bacterial suspensions (100 µl) were spread uniformly on 20 ml MH agar Petri plate (90 mm) after which paper discs (6 mm diameter) impregnated with 1 mg individual plant extracts are placed on inoculated Mueller-Hinton Agar plate. Zone of inhibition is measured in millimeters after incubation at 35 °C for 18-20 hours. Streptomycin susceptibility discs (10 µg) and respective solvent impregnated discs were used as positive and negative controls.

Broth microdilution test

Determination of minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of the plant extracts against the test organisms was carried out by broth microdilution method as recommended by national committee for clinical laboratory standards [17]. In short, broth micro dilution method was performed using microtitre plates. Stock solution of individual plant extracts were prepared in 2 fold dilution starting from the concentration of 20 mg/ml (1:2 to 1:128). The microtitre plate was prepared with 100 µl of inoculums containing 10⁸ cfu bacterial suspensions in every well. To each of the well, 100 µl of plant extracts from each concentration of the stock solution was added. Negative control contained the inoculums without extracts and the positive control contained streptomycin replacing the plant extracts. Microtitre plates were incubated at 35°C for 18-20 hours. After incubation, 10 µl from each well containing culture medium was inoculated in MHA plate. These MHA plates were incubated at 35°C for 18-20 hours and the number of surviving organisms (colonies) was observed. The MHA plate with no visible bacterial growth indicated 99% killing of the original inoculums and was noted as the MBC. MIC was determined by plotting a graph with number of colonies against concentration of plant extract in logarithmic values.

Synergistic effect

Polyherbal mix of *T. procumbens*, *L. camara*, *E. hirta* and *T. peruviana* was prepared at 1:1:1:1 ratio [18] containing the plant extracts. The synergistic effect of polyherbal mix of petroleum ether, acetone and methanol plant extracts were tested for anti-oxidant, anti-tyrosinase and anti-bacterial activities using DPPH assay, mushroom tyrosinase inhibition method and broth microdilution method.

Development of formulation and preservative efficacy study

A skin care cream was developed with the ingredients such as stearic acid, polyglycerly-3 methyl glucose distearate, PEG-100, almond oil and glycerin. The polyherbal extract was uniformly mixed into the cream with a blender. Preservative efficacy study of the skincare formulation was performed by challenging it with test microorganisms: *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* as per the methodology of British Pharmacopoeia 2002.

Statistical analysis

The experiment was a completely randomized design with three replicates. Data were subjected to analysis of variance (ANOVA) and means were calculated with standard error using Turkey multiple comparison test using SPSS version 13. P values < 0.05 were regarded as significant.

RESULTS AND DISCUSSION

Anti-oxidant activity

DPPH scavenging percentage of the positive control was found to be 75.82%, 76.11% and 68.79% in petroleum ether, acetone and methanol. Methanol extract of *Euphorbia hirta* (87.61%) showed the highest DPPH scavenging activity surpassing scavenging activity of that of positive control. The lowest DPPH scavenging activity is seen in the petroleum ether extract of *Thevetia peruviana* (9.51%). FRAP value of positive control was found to be 14.87 µMol/g, 326.84 µMol/g and 101.57 µMol/g. The highest FRAP value of 146.96 µMol/g, 417.90 µMol/g and 122.93 µMol/g is seen in *Euphorbia hirta* amongst the selected plants in petroleum ether, acetone and methanol plant extracts respectively. The least amount of antioxidant activity has been exhibited by petroleum ether extract of *Tridax procumbens* (8.13 µMol/g).

A correlation analysis was carried out using SPSS (version 13.0) to associate the scavenging property of the antioxidant (analysed using DPPH assay) and ferric reducing anti-oxidant property (analyzed using FRAP assay) of the individual extracts and there was found to be a strong positive correlation that is analogous to the other researcher's findings [19]. The correlation coefficient was found to be 0.944, 0.935 and 0.975 for petroleum ether, acetone and methanol plant extracts respectively.

Anti-tyrosinase activity

It was observed that the highest anti-tyrosinase property possessing plant extract is acetone extract of *Euphorbia hirta* (0.91 KAE). Lowest anti-tyrosinase activity was noted in petroleum ether extracts of *Tridax procumbens* (0.31 KAE). Tyrosinase inhibition by the plant extracts increased in the following order: acetone extracts > methanol extracts > petroleum ether extracts.

Anti-bacterial activity

Disc diffusion test

Plant extracts of petroleum ether, acetone and methanol were tested against a gram positive and gram negative bacteria. The data showed that the test organisms were highly susceptible to streptomycin with a zone of inhibition of more than 28 cm for gram positive and 17 cm for gram negative bacteria. Gram-positive bacteria were more susceptible due to the outer lipid layer. Of all the plant extracts, only acetone extracts showed zone of inhibition. Since, petroleum ether plant extracts and methanol plant extracts did not show any zone of inhibition, acetone plant extracts were alone taken for further analysis in order to determine MIC and MBC values.

Broth microdilution test

Acetone extracts of four selected plants were subjected to broth micro dilution test against two gram positive bacteria - *Micrococcus luteus*, *Staphylococcus aureus* and two gram negative bacteria - *Pseudomonas aeruginosa*, *Escherichia coli*. It was found that leaves of *Tridax procumbens* (0.165 mg/ml) extract possess the highest anti-bacterial property when compared to the rest of the plant extracts. *Thevetia peruviana* (5.31 mg/ml) plant extract showed poor anti-bacterial property which is nearly 35 times lesser than the activity of *Tridax procumbens*.

Synergistic effect

Anti-tyrosinase activity in individual plant extracts versus polyherbal mix

From the Fig. 1, it could be observed that polyherbal mix of four plants showed no notable enhancement of antityrosinase activity when compared to other individual plant extracts. Hence, no significant synergistic effect could be reported for anti-tyrosinase activity.

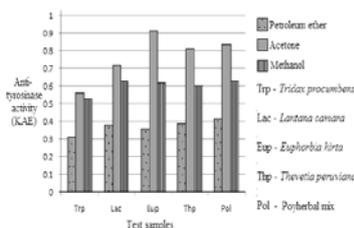


Fig. 1: Tyrosinase inhibition of individual plant extracts versus polyherbal mix

Table 1: Synergistic effect of acetone plant extracts for antibacterial activity - MBC (mg/ml)

Plant	Microorganism	<i>M. luteus</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
<i>Tridax procumbens</i>		0.312	0.625	0.625	1.250
<i>Lantana camara</i>		0.625	1.250	2.500	5.000
<i>Euphorbia hirta</i>		1.250	2.500	5.000	5.000
<i>Thevetia peruviana</i>		10.00	10.00	20.00	20.00
Polyherbal mix		0.312	0.625	0.625	1.250
Control	Streptomycin	0.312	0.625	2.500	2.500

Table 2: Recovery of challenged microorganisms at different time intervals from skincare formulation

Type of organisms	Species	Recovery			
		48h	7d	14d	28d
bacteria	<i>Pseudomonas aeruginosa</i>	+	-	-	-
	<i>Escherichia coli</i>	-	-	-	-
	<i>Staphylococcus aureus</i>	-	-	-	-
fungi	<i>Candida albicans</i>	-	-	-	-
	<i>Aspergillus niger</i>	+	+	+	-

Anti-oxidant activity in individual plant extracts versus polyherbal mix

The anti-oxidant activity expressed by polyherbal mix was compared with that of individual plant extracts in Fig. 2. The bar chart clearly shows additive anti-oxidant activity of polyherbal mix which outwits the anti-oxidant activity of individual plant extracts from petroleum ether, acetone and methanol. Therefore, synergistic effect has been recorded for anti-oxidant activity

Anti-bacterial activity in individual plant extracts versus polyherbal mix

The anti-bacterial activity is presented in Table 1. From Table 1, we can infer that the polyherbal mix is effective as it requires less concentration of sample to kill 99% of the bacteria. Moreover, it could be found that the antibacterial activity expressed by the polyherbal mix is equivalent to the *Tridax procumbens* which is close to the positive control. Hence, synergistic effect has been observed

for anti-bacterial activity. This confirms the polyherbal mix as an effective antibacterial plant extract mixture.

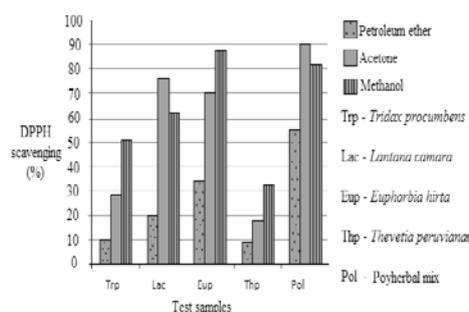


Fig. 2: Anti-oxidant activity of individual plant extracts versus polyherbal mix

Preservative efficacy study

After 48h, it was not possible to recovery the *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. After 7 days, it was not possible to recover *Pseudomonas aeruginosa* and after 14 days, *Aspergillus niger* could not be isolated (Table 2).

CONCLUSION

The anti-oxidant activity of plant extracts were evaluated using DPPH and FRAP assays which were found to be correlated. Antioxidant activity was observed to be the highest in methanol extract of *Euphorbia hirta* and acetone extract of *Euphorbia hirta* in DPPH and FRAP assays respectively. Acetone extracts of *Euphorbia hirta* showed the highest anti-tyrosinase activity. Anti-tyrosinase activity has been reported in *Tridax procumbens*, *Euphorbia hirta* and *Thevetia peruviana* for the first time in this study. Remarkable anti-bacterial activity was observed in acetone extracts of *Tridax procumbens*. Synergistic effect of the extracts in the polyherbal mix was observed for anti-oxidant and antibacterial activities. We have earlier reported the direct correlation between anti-tyrosinase activity and anti-oxidant activity of these plants and its possible utility in cosmetic and personal care industry for the dual benefits [20]. This study is unique as it reports possible 'triple' effects (anti-tyrosinase, anti-oxidant and antibacterial) of these selected plants. The polyherbal extract combination *Tridax procumbens*, *Lantana camara*, *Euphorbia hirta* and *Thevetia peruviana*, the only preservative in the skincare cream was able to reduce the load of the challenged Gram positive and Gram negative bacteria by 99% in 7 days and fungi (both mold and yeast form) in 28 days. Hence this combination would serve as a natural skin friendly preservative replacing the traditional synthetic preservatives.

The polyherbal extract can find its applications in cosmetics and hygiene/personal care products as a potential antimicrobial agent replacing synthetic compounds like triclosan, farnesol, derivatives of formaldehyde, etc which are known to cause human health problems and environmental pollution [21]. A polyherbal extract with multiple functional benefits would definitely bring down the levels of chemical 'do good' ingredients that are used for antimicrobial/preservative, anti-tyrosinase and anti-oxidant functions thereby making the formulation skin friendly.

Further, such a reduction of synthetic ingredients may also help in the reduction of the cost of such cosmetic formulations (consumer friendly) [21]. The data obtained might be considered sufficient and significant to pursue further research on the usage of selected plants in cosmetic/personal care industry and drug industry for its evidenced anti-oxidant, anti-tyrosinase and anti-bacterial activities. Further, research is also warranted to scrutinize the degree of toxicity of individual plant extracts or its combination.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Yirga G, Teferi M, Kasaye M. Survey of medicinal plants used to treat human ailments in Hawzen district, Northern Ethiopia. *Int J Biodiver Conser* 2011;3(13):709-14.
2. Tiwari P, Bimlesh K, Kaur M, Kaur G, Kaur H. Phytochemical screening and Extraction: a review. *Int Pharm Sci* 2011;1(1):98-106.
3. Gokulshankar S, Ranjith MS, Babu K, Deepa MA, Mohanty BK, et al. In Fairness in a natural way-Novel polyherbal ingredients inhibiting melanin synthesis and transfer Melanin: Biosynthesis, Functions and Health Effects (ISBN: 978-1-61942-019-9) Nova Publishers USA; 2012.
4. Chang, TS. An updated review of tyrosinase inhibitors. *International J Mol Sci* 2009;10(6):2440-75.
5. Palmer, Kitchin. Oxidative damage, skin aging and antioxidants and a novel antioxidant rating system. *J Drugs Dermatol* 2010;9(1):11-5.
6. Krishnamoorthy JR, Sumithira R, Gokulshankar S, Ranjith MS, Ranganathan S, Mohanty BK. Hair growth modulating effect of a novel herbal formulation. A rediscovery of traditional knowledge. *J Appl Cosmetol* 2010;28:147-51.
7. Miyazawa M, Oshima T, Koshio K, Itsuzaki Y, Jun Anzai. Tyrosinase inhibitor from from black rice bran. *J Agricultural Food Chem* 2003;51(24):6953-6.
8. Abdulla E, Raus RA, Jamal P. Extraction and Evaluation of Anti-bacterial Activity from Selected Plants. *Am Med J* 2012;3(1):27-32.
9. Saxena VK, Albert S. Beta-sitosterol-3-O-beta-D-xylopyranoside from the flowers of *Tridax procumbens* Linn. *J Chem Sci* 2005;117(3):263-6.
10. Raina R, Prawez S, Verma PK, Pankaj NK. Medicinal Plants and their Role in Wound Healing. *Vet Scan* 2008;3(1):1-7.
11. Kumar S, Malhotra R, Kumar D. *Euphorbia hirta*: its chemistry, traditional and medicinal uses, and pharmacological activities. *Pharmacognosy Rev* 2010;4:58-61.
12. Zibbu G, Batra A. GC-MS Analysis of the dessert plants of Apocynaceae family: *nerium oleander* and *Thevetia peruviana*. *Int J Pharm Res Development* 2011;3(10):49-62.
13. Kamkar A, Jebelli JA, Asadi F, Kamalinejad M. The antioxidative effect of Iranian Mentha pulegium extracts and essential oil in sunflower oil. *Food Chem Toxicology* 2010;48(7):1796-800.
14. Benzie IF, Strain JJ. "The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Anal Biochem* 1996;239(1):70-6.
15. Narisa K, Narong M, Charoen T. Screening of some tropical vegetables for anti-tyrosinase activity. *Pharm Health J* 2007;2(1):15-9.
16. Bauer AW, Kirby WM, Sherris JC. Antibiotic susceptibility testing by a standard single disc method. *Am J Clinical Pathology* 1996;45:493-6.
17. NCCLS. Performance Standards for antimicrobial susceptibility testing. (12th ed.) 2002.
18. Rhet DG, Haiying T, Steven S. Synergistic effects of antioxidants on the Oxidative stability of Soybean Oil – and Poultry Fat-Based Biodiesel. *J Am Oil Chemists's Soc* 2009;86:459-67.
19. Guo DJ, Cheng HL, Chan SW, Yu PH. Antioxidative activities and the total phenol contents of tonic Chinese medicinal herbs. *Inflamo pharmacology* 2008;16(5):201-7.
20. Sindhuja Priyadharsini R, Prabhakaran G, Gokulshankar S. Dual skin-care activity of selected herbs–Promising 'do-good' ingredients in Skin care formulations? *J Appl Cosmetol* 2013;31(2):113-22.
21. Mohanty BK, Gokulshankar S, Prabhmanju M, Ranjith MS, Ashutosh SR. Dandruff-A management strategy for one of the most chronic & commercially exploited skin conditions. Ed. A Mendez-Vilas Worldwide Research Efforts in the Fighting Against Microbial Pathogens: From Basic Research to Technological Developments (ISBN-10:1612336361;ISBN-13:9781612336367) BrownWalker Press, USA; 2012.