



Inhibition Test of Heme Detoxification (ITHD) as an Approach for Detecting Antimalarial Agents in Medicinal Plants

M. Mosaddegh¹, M. Irani¹, S. Esmaili^{1,2*}

¹Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran.

²Department of Traditional Pharmacy, School of Traditional Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Abstract

Background and objectives: There are several methods to assess the *in vitro* capability of heme inhibitory activity of antimalarial compounds; most of them require some specific equipment or toxic substances and sometimes the needed materials are not accessible. Regarding the necessity and importance of optimizing and standardizing experimental conditions, the present study has intended to improve the *in vitro* assessment conditions of the β -hematin formation inhibitory activity for screening herbal samples. **Methods:** Hemin, tween 20, and samples (9:9:2) were incubated in different conditions including: hemin concentration (30, 60, and 120 $\mu\text{g/mL}$), duration (4, 24, 48, and 72 h), pH of buffer (3.6, 4, 4.4, 4.8, and 5), and temperature (37 and 60 $^{\circ}\text{C}$) in 96-well plates. Also, a total of 165 plant extracts and fractions were tested in the most suitable conditions. **Results:** The reaction time and the incubation temperature were determined as the critical factors. The effective conditions for β -hematin formation were found to be 60 $^{\circ}\text{C}$ after 24 h incubation. In this method, proper correlations with respect to negative (69%) and positive (67%) predictive values were obtained in comparison with the anti-plasmodial assay. Antimalarial activities of *Pistacia atlantica*, *Myrtus communis*, *Pterocarya fraxinifolia*, and *Satureja mutica* were found to correlate significantly with inhibition of the heme detoxification assay. **Conclusion:** These results support a rapid, simple and reliable approach for selecting and identifying a number of herbs for further related antimalaria investigations.

Keywords: fever; heme detoxification; Iranian traditional medicine; malaria

Introduction

Malaria is an infectious disease caused by *Plasmodium* parasites. Approximately 3.3 billion people (half of the world's population) are at risk of malaria. According to the latest reports of WHO, there has been roughly 212 million malaria cases and an estimated 429000 malaria

deaths in 2015 [1].

The malaria parasite digests hemoglobin in vacuole into amino acids and heme [2]. Free heme is toxic for the parasite [3] and *Plasmodium* has got several detoxification mechanisms such as hemozoin formation, heme-binding proteins

*Corresponding author: sesmaeili@sbmu.ac.ir,

and degradation of free heme by H_2O_2 to protect itself from toxic heme [4,5]. Some drugs show antimalarial effects via inhibition of hemozoin formation which is the most important mechanism for detoxification [6,7]. β -hematin, which is identical to hemozoin, is a synthetic polymer that is used for malaria in vitro assessments [8,9]. Identifying compounds that inhibit β -hematin formation is an approach for detecting antimalarial drugs. To date, various methods in measuring β -hematin formation inhibitory activity have been published; most of them require specific equipment [10,11] or toxic substances [12], and sometimes the needed materials are not available [7,13]. Huy et al. described a simple colorimetric inhibition assay of heme crystallization [14]. Regarding the necessity and importance of optimizing and standardizing experimental conditions, the present study has intended to improve the in vitro assay conditions of the β -hematin formation inhibitory activity for herbal samples. Also, medicinal plants, which were traditionally used to treat fever in Iranian traditional medicine, were screened by inhibition test of heme detoxification (ITHD).

Material and Methods

Plant material

The selected plants were the most commonly used in Iranian traditional medicine for treating diseases, especially fever [15]. Most of the specimens were collected from different places of Iran and identified at Traditional Medicine and Materia Medica Research Center (TMRC), Tehran, Iran; others were purchased from herbal market. Voucher specimens were deposited at the Herbarium of TMRC. The selected herbal samples have been mentioned in table 1.

Chemicals and reagents

Chloroquine diphosphate (Sigma, USA); DMSO (dimethyl sulfoxide), tween 20, sodium acetate, acetic acid (Merck, Germany); hemin (Alfa Aesar, Germany), were used in this study. The solvents for extraction and fractionation were

provided from Mojalali Co. (Iran).

Extraction and fractionation

For preparing the crude extracts, 10 g of the dried powdered plants were macerated with constant shaking using 100 mL methanol for 24 h at room temperature. The fractions were prepared by macerating the dried powder or the residue of the plants in the solvent for 24 h at room temperature. Petroleum ether, chloroform and methanol were used successively for fractionation. The extracts and fractions were filtered and evaporated under vacuum. The dried extracts and fractions were stored at 4 °C.

Inhibition test of heme detoxification (ITHD)

The ITHD method was inspired from that previously described by Huy et al. [14]. In this method, some modifications were applied including: hemin concentration (30, 60, and 120 $\mu\text{g}/\text{mL}$), duration (4, 24, 48, and 72 h), pH (3.6, 4, 4.4, 4.8, and 5), and temperature (37 and 60 °C). The procedure for inhibition test was as follows; hemin was dissolved in dimethyl sulfoxide (DMSO). The solution was diluted freshly to 60 $\mu\text{g}/\text{mL}$ with 1 M acetate buffer (pH 4.8). Tween 20 was diluted to 0.012 g/L with distilled water. Diluted hemin, tween 20, and samples which were solved by DMSO, were distributed in each well of a 96-well plate with ratio of 9:9:2, respectively, in triplicate. Final tested concentration of samples in each well was 200 $\mu\text{g}/\text{mL}$. Also a control was prepared in triplicate under the same conditions, in the absence of hemin. The use of controls allowed to get rid of the residual interfering absorbance caused by the matrix of the sample. In negative control wells, the solvent (DMSO) used to solubilize the samples was added. Chloroquine diphosphate was used as the positive control. The plates were incubated at 60 °C for 24 h to allow completing the reaction. Finally, the absorbance was recorded with an ELISA reader at 405 nm. The results were calculated and expressed as percentage of heme detoxification inhibition.

Determination of performance of ITHD

This method was tested using a group of forty-five samples (table 1) most of them have been reported to show anti-plasmodial effects against *Plasmodium falciparum* [15-16]. The ITHD assay performance was evaluated by calculating the positive predictive value (PPV) and negative predictive value (NPV) according to the following equations:

$$PPV = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false positives}}$$

$$NPV = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false negatives}}$$

Results and Discussion

The aim of the current study was to develop a method to detect compounds with potential antimalarial activity. In the present study the

ITHD method was modified from that previously described by Huy et al. [14]. The critical parameters identified for reaction were the reaction time (24 h) and the incubation temperature (60 °C). Chloroquine diphosphate is known to act as an inhibitor of heme polymerization. Under the conditions of ITHD, there was a direct relation between chloroquine concentration and percentage of inhibition of heme detoxification, and therefore the value of IC₅₀ was obtained to be 14.1 ± 0.2 μM. Table 1 has presented the results of heme detoxification inhibition in forty-five herbal samples.

To evaluate the practical performance of the assay, we compared the results of inhibition test of heme detoxification with their anti-plasmodial activity (table 2) [15,16]. If percentage of heme detoxification inhibition was more than 90%, the assay was considered as positive whereas values less than 90% indicated a negative result.

Table 1. The selected herbal samples investigated for inhibition test of heme detoxification (ITHD)

| No. | Scientific name | Family | Plant part | Voucher number | Locality ^a | ITHD assay |
|-----|--|----------------|------------------------------------|----------------|-----------------------|------------|
| 1 | <i>Astrodaucus orientalis</i> (L.) Drude. | Apiaceae | Fruit-bearing & Flowering branches | TMRC 1276 | Tehran | - |
| 2 | <i>Berberis crataegina</i> DC. | Berberidaceae | Fruit-bearing branches | TMRC 1289 | Khorassan | - |
| 3 | <i>Biebersteinia multifida</i> DC. | Geraniaceae | Tuber | TMRC 486 | Khorassan-e-Shomali | - |
| 4 | <i>Bryonia aspera</i> Stev. ex Ledeb. | Cucurbitaceae | Tuber | TMRC 252 | Golestan | + |
| 5 | <i>Buxus hyrcana</i> Pojark. | Buxaceae | Flowering branches | TMRC 1161 | Gilan | - |
| 6 | <i>Caccinia macranthera</i> (Banks & Sol.) Brand | Boraginaceae | Root | TMRC 510 | Golestan | - |
| 7 | <i>Capparis spinosa</i> L. | Capparaceae | Flowering branches | TMRC 1295 | Golestan | - |
| 8 | <i>Centaurea bruguierana</i> (DC.) Bornm. | Asteraceae | Fruit-bearing branches | TMRC 1291 | Khorassan-e-Shomali | - |
| 9 | <i>Centaurea golestanica</i> Akhiani & Wagenitz | Asteraceae | Aerial Part | TMRC 730 | Golestan | - |
| 10 | <i>Cephalanthera caucasica</i> Kranzl. | Orchidaceae | Flowering branches | TMRC 1288 | Mazandaran | - |
| 11 | <i>Chenopodium botrys</i> L. | Chenopodiaceae | Fruit-bearing branches | TMRC 1331 | Tehran | - |
| 12 | <i>Colutea persica</i> Boiss. | Fabaceae | Fruit-bearing branches | TMRC 1253 | Mazandaran | - |
| 13 | <i>Dorema hyrcanum</i> Kos-Pol. | Apiaceae | Root | TMRC 414 | Golestan | - |
| 14 | <i>Epilobium hirsutum</i> L. | Onagraceae | Aerial Part | TMRC 1694 | Mazandaran | - |
| 15 | <i>Erodium oxyrhynchum</i> M. B. | Geraniaceae | Fruit-bearing & Flowering branches | TMRC 1277 | Tehran | - |
| 16 | <i>Ferula oopoda</i> (Boiss. & Buhse) Boiss. | Apiaceae | Leaves | TMRC 970 | Golestan | - |

Mosaddegh M. et al.

Table 1. Continued

| No. | Scientific name | Family | Plant part | Voucher number | Locality ^a | ITHD assay |
|-----|---|------------------|------------------------------------|----------------|---------------------------|------------|
| 17 | <i>Ferula oopoda</i> (Boiss. & Buhse) Boiss. | Apiaceae | Root | TMRC 970 | Golestan | - |
| 18 | <i>Ferula szowitziana</i> DC. | Apiaceae | Root | TMRC 965 | Golestan | - |
| 19 | <i>Ficus carica</i> L. | Moraceae | Fruit-bearing branches | TMRC 1338 | Mazandaran | - |
| 20 | <i>Glaucium oxylobum</i> Boiss. & Buhse | Papaveraceae | Flowering branches | TMRC 1283 | Gilan | - |
| 21 | <i>Glycyrrhiza glabra</i> L. | Fabaceae | Young branch | TMRC 1170 | Gilan | - |
| 22 | <i>Leontice leontopetalum</i> L. | podophyllaceae | Tuber | TMRC 1287 | Khorassan-e-Shomali | - |
| 23 | <i>Linaria pyramidata</i> Lam. | Scrophulariaceae | Flowering branches | TMRC 1293 | Khorassan | - |
| 24 | <i>Marrubium vulgare</i> L. | Lamiaceae | Flowering branches | TMRC 1286 | Mazandaran | - |
| 25 | <i>Matricaria chamomilla</i> L. | Asteraceae | Aerial Part | HMS 330 | Tehran | - |
| 26 | <i>Minuartia lineata</i> (Boiss.) Bornm. | Caryophyllaceae | Fruit-bearing branches | TMRC 1279 | Mazandaran | - |
| 27 | <i>Myrtus communis</i> L. | Myrtaceae | Flowering branches | TMRC 1169 | Gilan | + |
| 28 | <i>Ononis spinosa</i> L. | Fabaceae | Aerial Part | TMRC 1813 | Azarbajjan-e-Sharghi | - |
| 29 | <i>Otostegia persica</i> (Burm.) Boiss. | Lamiaceae | Aerial Part | HMS 329 | Sistan-va-Baluchestan | - |
| 30 | <i>Otostegia michauxii</i> Briq. | Lamiaceae | Aerial Part | TMRC 1152 | Fars | - |
| 31 | <i>Paliurus spina-christi</i> Mill. | Rhamnaceae | Flowering branches | TMRC 1282 | Mazandaran | - |
| 32 | <i>Papaver bracteatum</i> Lindl. | Papaveraceae | Flowering branches | TMRC 1297 | Ardebil | - |
| 33 | <i>Parrotia persica</i> (DC.) C.A. Mey. | Hamamelidaceae | Bark | TMRC 1281 | Golestan | - |
| 34 | <i>Perovskia abrotanoides</i> Karel. | Lamiaceae | Aerial Part | TMRC 801 | Khorassan | - |
| 35 | <i>Phytolacca americana</i> L. | Phytolaccaceae | Aerial Part | TMRC 1154 | Mazandaran | - |
| 36 | <i>Pistacia atlantica</i> (F. & C. A. Mey) Rech. F. | Anacardiaceae | Young branch | TMRC 1271 | Gilan | + |
| 37 | <i>Pterocarya fraxinifolia</i> (Lam.) Spach | Juglandaceae | Fruit-bearing branches | TMRC 1167 | Mazandaran | + |
| 38 | <i>Pteropyrum aucheri</i> Jaub. & Spach | Polygonaceae | Fruit-bearing & Flowering branches | TMRC 1285 | Qazvin | + |
| 39 | <i>Rhamnus cornifolia</i> Boiss. and Hohen. | Rhamnaceae | Fruit-bearing branches | TMRC 1274 | Kohgiluyeh-va-Boyer-Ahmad | - |
| 40 | <i>Roemeria refracta</i> DC. | Papaveraceae | Flowering branches | TMRC 1292 | Khorassan | - |
| 41 | <i>Satureja mutica</i> Fisch. & C. A. Mey. | Lamiaceae | Aerial Part | TMRC 864 | Golestan | + |
| 42 | <i>Scutellaria multicaulis</i> Boiss. | Lamiaceae | Flowering branches | TMRC 1273 | Kohgiluyeh-va-Boyer-Ahmad | - |
| 43 | <i>Smilax excelsa</i> L. | Liliaceae | Aerial Part | TMRC 1511 | Mazandaran | - |
| 44 | <i>Tamarix aralensis</i> Bunge | Tamaricaceae | Fruit-bearing & Flowering branches | TMRC 1284 | Gilan | - |
| 45 | <i>Vitex pseudo-negundo</i> (Hauskn.) Hand.- Mzt. | Verbenaceae | Fruit-bearing & Flowering branches | TMRC 1153 | Fars | - |

^a The province in Iran;

-: percentage of heme detoxification was less than 90%; +: percentage of heme detoxification was more than 90%

Extracts that inhibited *Plasmodium* growth by less than 50% at the concentration of 25 µg/ mL ($IC_{50} > 25$ µg/ mL) were grouped as being not significantly growth inhibitors. Although their antimalarial activity had been previously studied [15,16] to the best of our knowledge, their mode of action had not been published.

Table 2. Correlation between ITHD and the in vitro *Plasmodium falciparum* growth inhibition assay

| | | In vitro <i>P. falciparum</i> growth inhibition assay | |
|------|----------|---|-------------------------------------|
| | | Positive ($IC_{50} \leq 25$ µg/mL) | Negative ($IC_{50} > 25$ µg/mL) |
| ITHD | Positive | 4 | 2 |
| | Negative | 12 | 27 |

PPV value of 67% and NPV of 69% pointed out the good performance of the test in respect to the in vitro *P. falciparum* assay. Vargas *et al* reported good correlations in their method in respect with extracts PPV of 50% [17]. *Pistacia atlantica*, *Myrtus communis*, *Pterocarya fraxinifolia* and *Satureja mutica* showed significant inhibition through plasmodium growth and heme detoxification. False negative compounds may show their anti-plasmodial activity via other mode of actions. However, this method is a good predictor for screening antimalarial activity in compounds capable of interacting with heme. The advantage of ITHD assay is its simplicity and low cost for screening large samples, because it can be performed with basic laboratory equipment without handling *P. falciparum*. A total number of 120 fractions from 40 plants were screened; 10% of fractions were heme inhibitors, and the methanol fractions were more effective compared to others. The results showed that the methanol fraction of *Glycyrrhiza glabra*, *Pistacia atlantica*, *Myrtus communis*, *Parrotia persica*, *Pteropyrum aucheri*, *Ferula oopoda*, *Roemeria refracta*, and *Tamarix aralensis*; the chloroform fraction of *Astrodaucus orientalis*, *Otostegia persica*; and the petroleum ether fraction of *Otostegia michauxii*, *Perovskia abrotanoides* could inhibit heme detoxification. The present data demonstrated the action mode of three plants with anti-plasmodial activity

including *G. glabra*, *P. atlantica* and *M. communis*.

The crude extract of *G. glabra* (licorice) did not show any inhibitory activity of heme detoxification at 200 µg/ mL, whereas it had shown anti-plasmodial activity against resistant strain K1 and antimalarial activity in vivo [15]; however, its methanol fraction showed considerable activity by ITHD. The use of a preliminary cleaning step removed a large part of the interfering compounds. This purification has, therefore, led to a significant improvement of the correlation between the hematin assay and the *P. falciparum* growth inhibition assay justifying the cost and labor related to this additional purification step [17]. Chen *et al.* reported licochalcone A isolated from Chinese licorice roots, inhibiting the in vitro growth of both chloroquine-susceptible (3D7) and chloroquine-resistant (Dd2) *P. falciparum* strains; they demonstrated that licochalcone A might be developed into a new antimalarial drug [18].

According to the results of the study, total extract and methanol fraction of *Pistacia atlantica* showed inhibitory effects on heme detoxification. Orhan *et al.* reported that the leaf extract was active against *P. falciparum* among thirteen lipophilic extracts prepared from various parts of *Pistacia vera* L. [19]. Adams *et al.* screened some plant extracts for antimalarial activity and found an ethyl acetate extract of *P. atlantica* was active. With subsequent isolation methods and structure elucidation, flavone 3-methoxycarpachromene was identified as the active substance [20]. So it may be considered as a selective plant for further antimalarial assays.

In our survey, flowering branches' extract of *M. communis* (myrtle) showed inhibitory effects. Also, methanol fraction of the species was effective. Milhau *et al.* showed that essential oils of leaves and stems of *M. communis* inhibited *P. falciparum* [21] and Naghibi *et al.* reported anti-plasmodial activity of *M. communis*, using the parasite lactate dehydrogenase assay; The IC_{50} values of *M. communis* extract were 35.44 and 0.87 µg/mL against K1 and 3D7 strains of *P.*

falciparum, respectively. Also the myrtle extract showed a significant inhibition in in vivo antimalarial assay [22]. These results support an approach for identifying plant-based agents with significant antimalarial effect.

Acknowledgements

This study was supported by grant (No. 90/1596) from Shahid Beheshti University of Medical Science. We are grateful to Saeedeh Ghafari and Azadeh Ghiaee for partial assistance.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

References

- [1] World Health Organization. *Fact Sheet: World Malaria Report*. [Accessed 2017]. Available from: <http://www.who.int/malaria/media/world-malaria-report-2016/en/>.
- [2] Krugliak M, Zhang F, Ginsburg H. Intraerythrocytic *Plasmodium falciparum* utilizes only a fraction of the amino acids derived from the digestion of host cell cytosol for the biosynthesis of its proteins. *Mol Biochem Parasitol*. 2002; 119(2): 249-256.
- [3] Schmitt TH, Frezzatti WA, Schreier S. Hemin-induced lipid membrane disorder and increased permeability: a molecular model for the mechanism of cell lysis. *Arch Biochem Biophys*. 1993; 307(1): 96-103.
- [4] Slater AF, Cerami A. Inhibition by chloroquine of a novel heme polymerase enzyme activity in malaria trophozoites. *Nature*. 1992; 355(6356): 167-169.
- [5] Campanale N, Nickel C, Daubenberger CA, Wehlan DA, Gorman JJ, Klonis N, Becker K, Tilley L. Identification and characterization of heme-interacting proteins in the malaria parasite, *Plasmodium falciparum*. *J Biol Chem*. 2003; 278(30): 27354-27361.
- [6] Kumar S, Guha M, Choubey V. Antimalarial drugs inhibiting hemozoin (β -hematin) formation: a mechanistic update. *Life Sci*. 2007; 80(9): 813-828.
- [7] Sullivan DJ, Gluzman IY, Russell DG, Goldberg DE. On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci*. 1996; 93(21): 11865-11870.
- [8] Slater AF, Swiggard WJ, Orton BR, Flitter WD, Goldberg DE, Cerami A, Henderson GB. An iron-carboxylate bond links the heme units of malaria pigment. *Proc Natl Acad Sci*. 1991; 88(2): 325-329.
- [9] Blauer G, Akkawi M. On the preparation of β -haematin. *Biochem J*. 2000; 346(2): 249-250.
- [10] Deharo E, Garcia RN, Oporto P, Gimenez A, Sauvain M, Jullian V, Ginsburg H. A non-radiolabelled ferriprotoporphyrin IX biomineralisation inhibition test for the high throughput screening of antimalarial compounds. *Exp Parasitol*. 2002; 100(4): 252-256.
- [11] Tripathi AK, Khan SI, Walker LA, Tekwani BL. Spectrophotometric determination of *de novo* hemozoin/beta-hematin formation in an *in vitro* assay. *Anal Biochem*. 2004; 325(1): 85-91.
- [12] Ncokazi KK, Egan TJ. A colorimetric high-throughput beta-hematin inhibition screening assay for use in the search for antimalarial compounds. *Anal Biochem*. 2005; 338(2): 306-319.
- [13] Kurosawa Y, Dorn A, Kitsuji-Shirane M, Shimada H, Satoh T, Matile H, Hofheinz W, Masciadri R, Kansy M, Ridley RG. Hematin polymerization assay as a high-throughput screen for identification of new antimalarial pharmacophores. *Antimicrob Agents Chemother*. 2000; 44(10): 2638-2644.
- [14] Huy NT, Uyen DT, Maeda A, Xuan Trang DT, Oida T, Harada S, Kamei K. Simple colorimetric inhibition assay of heme crystallization for high throughput screening of antimalarial compounds. *Antimicrob Agents Chemother*. 2007; 51(1): 350-353.
- [15] Esmaeili S, Naghibi F, Mosaddegh M,

- Sahranavard Sh, Ghafari S, Noor Rain A. Screening of anti-plasmodial properties among some traditionally used Iranian plants. *J Ethnopharmacol.* 2009; 121(3): 400-404.
- [16] Naghibi F, Ghafari S, Esmaeili S, Jenett-Siems K. Naghibione a novel sesquiterpenoid with anti-plasmodial effect from *Dorema hyrcanum* Koso-Pol. root, a plant used in traditional medicine. *Iran J Pharm Res.* 2015; 14(3): 961-968.
- [17] Vargas S, Ndjoko Ioseta K, Hayb AE, Ioseta JR, Wittlinc S, Hostettmanna K. Screening medicinal plants for the detection of novel antimalarial products applying the inhibition of β -hematin formation. *J Pharm Biomed Anal.* 2011; 56(5): 880-886.
- [18] Chen M, Theander TG, Christensen SB, Hviid L, Zhai L, Kharazmil A. Licochalcone A, a new antimalarial agent, inhibits *in vitro* growth of the human malaria parasite *Plasmodium falciparum* and protects mice from *P. yoelii* infection. *Antimicrob Agents Chemother.* 1994; 38(7): 1470-1475.
- [19] Orhan I, Aslan M, Sener B, Kaiserb M, Tasdemir D. *In vitro* antiprotozoal activity of the lipophilic extracts of different parts of Turkish *Pistacia vera* L. *Phytomedicine.* 2006; 13(9-10): 735-739.
- [20] Adams M, Plitzko I, Kaiser M, Brun R, Hamburger M. HPLC-profiling for anti-plasmodial compounds -3-methoxycarpachromene from *Pistacia atlantica*. *Phytochem Lett.* 2009; 2(4): 159-162.
- [21] Milhau G, Valentina A, Benoita F, Mallie M, Bastide JM, Pelissier Y, Bessiere JM. *In vitro* antimalarial activity of eight essential oils. *J Essent Oil Res.* 1997; 9(3): 329-333.
- [22] Naghibi F, Esmaeili S, Noor Rain A, Nateghpour M, Taghvai M, Kamkar S, Mosaddegh M. *In vitro* and *in vivo* antimalarial evaluations of myrtle extract, a plant traditionally used for treatment of parasitic disorders. *Bio Med Res Int.* 2013; Article ID 316185.