Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

In vitro Antitoxoplasmal Activity of Some Medicinal Plants

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Submitted: 21-12-2018 Revised: 22-01-2019 Published: 19-09-2019

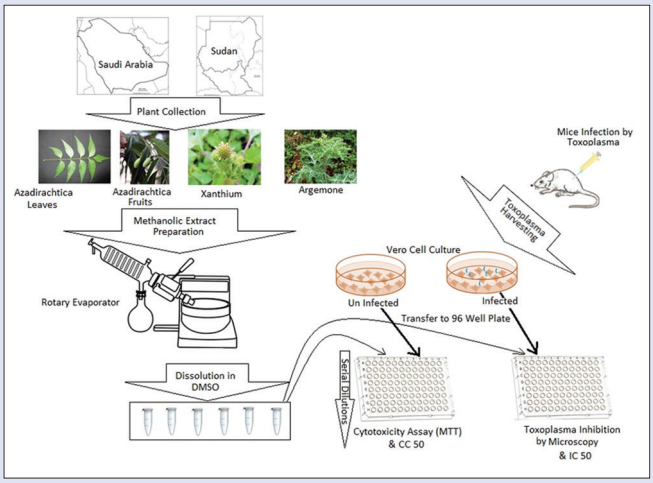
ABSTRACT

Background: Toxoplasmosis is a serious zoonotic protozoal disease that is distributed worldwide and can infect almost all warm-blooded animals, including humans. In most cases, it is asymptomatic, but in immunocompromised individuals, it is associated with severe neurological and gastrointestinal disorders. A previous serological prevalence investigation in Saudi Arabia indicated that the disease prevalence ranged between 25% and 51% in various areas. Recommended commercial drugs cannot achieve 100% clearance due to side effects. Hence, the development of new safe and affordable drugs is an important goal. **Aim:** In the present study, extracts from the leaves and fruit of *Azadirachta indica* A. Juss. collected from different areas, along with two other medicinal plants (*Argemone mexicana* L. and *Xanthium brasilicum* Vell.) with established antiprotozoal activity, will be evaluated for antitoxoplasmal activity using an *in vitro* technique. **Materials and Methods:** All plants were extracted with 100% methanol and examined for activity against the *Toxoplasma gondii* RH strain via an intracellularly invaded Vero cell line with calculated inhibition percentages. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay against the Vero cell line was used for cytotoxic evaluation, followed by selectivity index (SI) calculation. **Results:** *X. brasilicum* exhibited the best antitoxoplasmal activity with an IC_{50} of 7.19 μg/ml, followed by the *A. indica* fruits collected from Qassim, Kingdom of Saudi Arabia, and leaves collected from Central Sudan with an IC_{50} of 17.26 and 18.43 μg/ml, respectively. The best SI was obtained from the leaves of *A. indica* (6.28) collected from Sudan. **Conclusion:** Although *X. brasilicum* proved to have very potent antitoxoplasmal activity, the cytotoxicity was also very high, so the isolation of active compounds is highly recommended.

Key words: *Argemone*, *Azadirachta*, *Toxoplasma gondii*, *Xanthium*

SUMMARY

- Toxoplasmosis is a serious zoonotic protozoal disease that is distributed worldwide and can infect almost all warm-blooded animals, including humans, and associated with severe neurological and gastrointestinal disorders. A study in Saudi Arabia revealed that the disease prevalence ranged between 25% and 51% and that the development of new safe and affordable drugs is desirable as recommended commercial drugs are associated with many side effects. The present study aimed to evaluate the medicinal plants of different origins for their antitoxoplasmal activity. For this, the plants were extracted with methanol and examined for their percentage inhibition of *Toxoplasma gondii* RH strain which was maintained on Vero cell line. The cytotoxicity of the extracts was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *Xanthium brasilicum* exhibited the best antitoxoplasmal activity with an IC_{50} of 7.19 μg/ml but with high cytotoxicity; this was followed by the *Azadirachta indica* fruits collected from Qassim, Kingdom of Saudi Arabia, and leaves collected from Central Sudan with an IC_{50} of 17.26 and 18.43 μg/ml, respectively. The best selectivity index was obtained from the leaves of *A. indica* collected from Sudan (6.28). Potent active ingredients from *X. brasilicum* are recommended to be isolated because of its higher cytotoxicity.



Abbreviations used: MTT: 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; CNS: Central nervous system; AIDS: Acquired immune deficiency syndrome; DHFR: Dihydrofolate reductase; RPMI: Roswell Park Memorial Institute; FBS: Fetal bovine serum; OECD: Organization for Economic Co-operation and Development; DMSO: Dimethyl sulfoxide; ANOVA: Analysis of variance; SPSS: Statistical Package for the Social Sciences; LSD: Least significant difference; SI: Selectivity index; KSA: Kingdom of Saudi Arabia.

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DOI: 10.4103/pm.pm_646_18

Access this article online
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INTRODUCTION

Toxoplasmosis is caused by an Apicomplexa protozoan known as *Toxoplasma gondii*. It is cosmopolitan and zoonotic and can infect all mammals, but felids are the definitive host. The parasite changes between the tachyzoite and bradyzoite forms to invade muscular and nervous tissues.[1]

The prevalence of *T. gondii* infections in humans is very high, but its worldwide distribution is varying. Higher infection rates were reported in Central and South America and Europe (50%–80%), whereas

Cite this article as: Al Nasr IS, Koko WS, Khan TA, Elghazali GE. *In vitro* antitoxoplasmal activity of some medicinal plants. Phcog Mag 2019;15:568-72.

North America has the lowest prevalence of 1% in Alaska. In the United Kingdom, 0.35 million persons are infected annually.[2]

The disease is characterized by severe symptoms that can be potentially life threatening/fatal in immunocompromised people, leading to serious health problems including retinochoroiditis and central nervous system (CNS) symptoms in reactivated infections. Toxoplasmosis is the major cause of mortality for acquired immune deficiency syndrome-infected individuals. The progression of toxoplasmosis includes headache, lethargy, ataxia, and mental confusion and could also result in coma due to the necrosis of the thalamus.[3]

T. gondii infection in pregnant women is a primary concern as it increases the risk of miscarriage, and newborns may develop retinochoroiditis or CNS lesions.[4]

The most commonly used chemotherapies (sulfadiazine and pyrimethamine) for the treatment of toxoplasmosis have limited beneficial effects, particularly when the parasite is actively multiplying. Moreover, they usually fail to completely cure the infection. The parasites have developed multiple drug resistance, and these drugs are also contraindicated during pregnancy due to toxicity to the fetus.[5] Therefore, the search for new, naturally derived anti-Toxoplasma agents is a high priority.

Medicinal plants have long been used worldwide for the treatment of many parasitic diseases such as malaria, leishmaniasis, and leprosy Previously discovered antimalarial compounds isolated from medicinal plants (e.g., quinine and artemisinin) heralded the great potential of traditional plants, already used by mankind for centuries, as a source of new drugs against parasites, which has been confirmed by various articles.[6,7] For example, *Argemone mexicana* L. has previously been studied for its chemical constituents showing that most of the isolated compounds belong to the class of alkaloids. The other constituents include terpenoids, flavonoids, phenolics, long-chain aliphatic compounds, and few aromatic compounds. *A. mexicana* also possesses biological activity including antimicrobial, anti-HIV, nematicidal, anthelmintic, and antifungal activities.[8]

Previously, *Azadirachta indica* A. Juss. collected from Ivory Coast of West African region had been investigated for its anti-*T. gondii* activity showing promising parasiticidal effects, but the study lacked cytotoxicity investigation of the plant extracts. A study of similar plant species of East African for both anti-toxoplasma and cytotoxicity effects will surely highlight its therapeutic potential and possible variation in biological activity due to regional differences.[9] Chemical constituents of *A. indica* include glycosides, alkaloids, tannins, flavonoids, terpenoids, saponin, reducing sugar, and volatile oils.[10] The plant extracts have earlier been reported to have antimalarial activities.[11] *X. brasilicum* Vell. extract of East African origin has shown to possess significant biological activity against *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi* (Chagas disease), *Leishmania donovani* (kala-azar), as well as *Plasmodium falciparum* (malaria tropica).[12] The phytochemical analysis of the plant also revealed the presence of bioactive sesquiterpene lactones pungiolide and dinor-xanthanlide with varying degree of antiparasitic activity.[12]

Although toxoplasmosis has a global distribution, it is more prevalent in Saudi Arabia due to the presence of a large number of cats (definitive host of *T. gondii*). In Saudi Arabia, there is no national systemic serological toxoplasmosis screening program, though some researchers have conducted studies on the seroprevalence in different regions of the kingdom. These studies indicated that the seroprevalence ranged between 25.0% and 51.4% in different regions of Saudi Arabia: Al-Qurain (25.0%), Al-Nereiyah (26.36%), Makah (29.4%–35.6%), Abha (31.6%), Jeddah (31.6%), Najran (31.9%), Riyadh (21%–38%), Al-Hofuf (39.4%), Al-Khobar (39.4%), Dammam (40%), Asser (41%), and Al-Hasa (51.4%).[13-27]

It is also reported that in Saudi Arabia, toxoplasmosis was more common in rural areas, which might be due to an increased exposure of the people to animals either in houses or on farms during work, while cat feces in garden soils or farms or eating unwashed fruits and vegetables poses a higher risk of infection.[28] Therefore, control measures for *T. gondii*, including proper sanitation and management of animals (livestock and cats), should be well communicated and practiced. Once a person is infected, effective treatment requires the use of drugs. An ideal anti-toxoplasma drug should have antiparasitic properties against different stages of the parasitic life cycle including penetration into cysts. There should also be effective penetration through the placenta, and the drug should be non-toxic and non-teratogenic to the fetus. However, none of the currently used drugs meets these criteria. Drugs such as sulfadiazine (sulfonamide) have significantly lower antiparasitic activity if not used along with pyrimethamine (dihydrofolate reductase inhibitor).[6] When pyrimethamine is used for *T. gondii* infection treatment, it suppresses bone marrow function and can cause neutropenia. Furthermore, these drugs result in folate depletion, which can have detrimental effects on early fetal development; therefore, this treatment cannot be used in congenital toxoplasmosis during the first trimester of pregnancy. The combination of sulfadiazine and pyrimethamine also increases the risk of kidney stones, allergic reactions, and other forms of hepatic or renal complications.[5] Although new drugs such as atovaquone, epiroprim (DHFR inhibitor), and fluoroquinolone are effective *in vitro* and *in vivo* against *T. gondii*, their usage in pregnancy is limited as their safety has not been established.[28]

In this study, the antitoxoplasmal activity of leaves and fruits of *A. indica* A. Juss. collected from different regions and different climate conditions was investigated. The plant has well-known insecticidal and antiparasitic activities.[9-11] Moreover, two medicinal herbs (*A. mexicana* L. and *X. brasilicum* Vell.), proven to have potent antiprotozoal activity,[29,30] were investigated for antitoxoplasmal activity and cytotoxicity.

MATERIALS AND METHODS

Plant collection and extract preparation

The leaves and fruits of *A. indica* (Alq and Afq) were collected from Ar Rass city, Qassim region in Central Saudi Arabia (25° 51′ 60″ N, 43° 29′ 60″ E) and from Central Sudan at Khartoum State (15° 33′ 5″ N, 32° 30′ 41″ E) (Als and Afs). Whole plants of *A. mexicana* (Am) and *X. brasilicum* (Xb) were collected from the banks of the river Nile near the city of Khartoum in Sudan (15° 33′ 6″ N, 32° 31′ 56.7″ E). The plants were identified and classified by Prof. Dr. Gamal E. Elghazali the major taxonomist from the College of Science and Arts in Ar Rass, Qassim University, Kingdom of Saudi Arabia (KSA), where the voucher specimens were deposited at the Department of Laboratory Sciences with reference numbers AZIN041116111, ARME041116112, and XABR041116113, respectively. The samples were dried in a shed and ground to a fine powder using a Tecator 1093 sample mill. The powdered plant material was extracted with methanol using an overnight maceration technique. The extract was then filtered and evaporated under reduced pressure using a rotary evaporator.

In vitro evaluation of the crude extracts for antitoxoplasmal activity

Parasite and host cell maintenance

Serial passages of Vero cells were used to maintain RH strain *T. gondii* tachyzoites that were later used for *in vitro* experimental infection.

Vero cells were cultured using Roswell Park Memorial Institute (RPMI) 1640 medium enriched by heat-inactivated 10% fetal bovine serum (FBS) in 75 cm^2 culture flasks incubated at 37°C in 5% CO_2. Serial passages

were used for maintaining the *T. gondii* tachyzoites (RH strain) in Vero cells that were cultured in RPMI 1640 medium supplemented with 2% FBS.

Effect of plant extracts against T. gondii intracellular invasion of cultured Vero cells (bioassay)

Vero cell culture was conducted in 96-well plates (5×10^3 cells/well in 200 µL of RPMI 1640 medium with 10% FBS) which were incubated at 37°C and 5% CO_2 for 1 day, and then, the medium was removed and the cells were washed with PBS (phosphate-buffered saline). RPMI 1640 medium with 2% FBS containing tachyzoites (RH strain) of *T. gondii*, at a parasite: cell ratio of 5:1, was then added. Following incubation at 37°C and 5% CO_2 for 3 h, cells were treated as described below:

- Control: RPMI 1640 medium
- Positive control: Medium + atovaquone (reference: 50 µg/ml, 25, 12.5, etc.)
- Experimental: Medium + plant extract (50 µg/ml, 25, 12.5, etc.).

After a 72-h incubation at 37°C and 5% CO_2, the cells were washed in PBS, fixed in 10% formalin, and stained with 1% toluidine blue. The cells were examined under an inverted photomicroscope to determine the infection index (number of cells infected of the 200 cells tested) of *T. gondii*. The following equation was used for calculating the percentage inhibition of the infection index.

$$\text{Inhibition}(\%) = \frac{\text{I Control} - \text{I Experimental}}{\text{I Control}} \times 100$$

Where I Control refers to the untreated cells' infection index and I Experimental refers to the extract-treated cells' infection index.

In vitro cytotoxicity assay

A tetrazolium salt colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was conducted as described in the OECD guidelines,[31] for confirming the safety or toxicity of the plant extracts against host cells and to determine the plant extract concentration that can be safely used without negatively affecting the cells' viability. Briefly, Vero cells were cultured in 96-well plates (5×10^3 cells/well/200 µL) for 24 h in RPMI 1640 medium with 10% FBS and 5% CO_2 at 37°C. Cells were washed with PBS and treated with atovaquone for 72 h (positive control) or plant extracts at varying concentrations (50 µg/ml, 25, 12.5, etc.) in the medium with 2% FBS. For the negative control, cells were treated with medium only with 2% FBS. The cells were incubated with the plant extract for 72 h, then the supernatant was removed, and 200 ml of plain RPMI 1640 medium with 20 ml of MTT (5 mg/ml) was added. The cells were incubated with MTT for 4 h. Next, the supernatant was removed by inverting the plates onto tissue papers, and then, 100 ml of DMSO was added to dissolve the water-insoluble formazan salt. This colorimetric reaction was performed using a FLUOstar OPTIMA spectrophotometer, and the reading was performed at 540 nm. Cytotoxic effects were expressed as the concentration that caused a 50% reduction in viable cells (CC_{50}) compared to the control cells (those exposed to culture medium alone, without extracts or reference drug).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences computer program to compare the differences in means between the treated and control groups through least significant difference (LSD), and $P \leq 0.5$ was considered statistically significant. The results are expressed as the mean ± standard error of the mean. Linear regression was applied for calculating IC_{50}.

RESULTS

Figure 1 indicates the activity of plant methanol extracts against *T. gondii in vitro*. All the extracts showed variable rates of parasitic inhibition for the highest concentration of 50 µg/ml with 100, 94.3, 80, 69.7, 68.5, and 53.7% inhibition for Xb, Afq, Afs, Als, Am, and Alq, respectively. This inhibition decreased gradually with concentration, and there was zero inhibition which was detected in case of 12.5 µg/ml for Alq and Am. However, Xb, Afs, Afq, and Am showed a different parasitic inhibition at the minimum concentration of 6.25 µg/ml with 52.4, 16.9, 10.6, and 9.7% inhibition, respectively. ANOVA with LSD for Xb indicated different levels of significance, from $P < 0.05$ at 25 µg/ml, which increased with decreasing concentration to $P < 0.01$ at 12.5 and 6.25 µg/ml when compared to the means of the other extracts. In addition, the IC_{50} values for parasitic inhibition were determined as 7.2, 17.3, 18.4, 21.8, 33.6, and 38.2 µg/ml for Xb, Afq, Als, Afs, Am, and Alq, respectively [Table 1]. Atovaquone was used as a positive control; it possessed 100% parasitic inhibition at all tested concentrations (6.25 up to 50 µg/ml).

Figure 2 displays the results of the MTT cytotoxic assay against Vero cells, which indicated that all extracts had varying levels of toxicity at the highest concentration (50 µg/ml) from 100% mortality in Xb, 72.9% Afs, 50.3% Afq, 47.4% Als, 23.2% Am, and 18.7% Alq. These results were proportional to the CC_{50} values, which were 15.7, 25.6, 42.6, 63.6, 86.2, and 115.8 µg/ml for Xb, Afs, Afq, Am, Alq, and Als, respectively, with the exception of the last three extracts due to their activity at lower concentrations [Table 1]. Atovaquone was used as a positive control, and it showed no cell mortality for all tested concentrations (6.25 up to 50 µg/ml).

The selectivity index (SI) for comparing the relationship between activity and toxicity effects was derived by the CC_{50} values rather than IC_{50} values, so the highest value obtained (6.3) was by Als, followed by Afq 2.7, Alq 2.3, Xb 2.2, Am 1.9, and Afs 1.2 [Table 1].

DISCUSSION AND CONCLUSION

Modern trends have directed discovery efforts for new antiparasitic agents toward natural products with particular emphasis on medicinal plants.[32] Although toxoplasmosis is a health problem that is distributed worldwide and is particularly serious and potentially fatal for immunocompromised people,[3] little progress has been made in the discovery of antitoxoplasmal medicinal plants, especially in the Middle East region. In the present study, the methanol extracts of six different parts from three plants (collected from KSA and Sudan) already proven to have antiparasitic effects were examined for their antitoxoplasmal activity against *T. gondii* RH strain *in vitro* for the first time. All plant

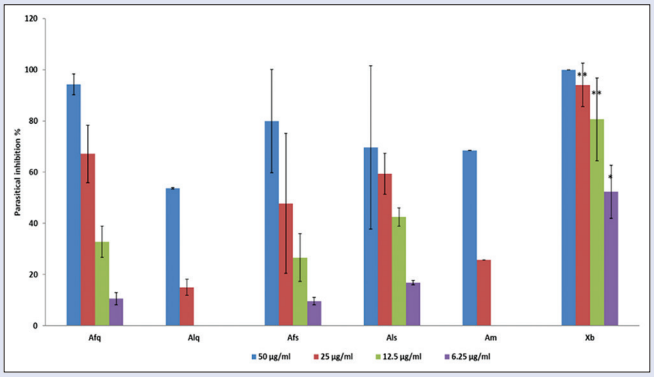

Figure 1: Toxoplasmal inhibition percentage of selected plant extracts. *$P < 0.05$, **$P < 0.01$

Table 1: Antitoxoplasmal IC_{50}, CC_{50} against Vero cells, and the selectivity index for selected plant methanol extracts

Name of plant extract	CC_{50} µg/ml cytotoxicity	IC_{50} µg/ml parasite inhibitory	SI (CC_{50}/IC_{50}) antitoxoplasmal activity
A. indica (KSA) fruits (Afq)	42.6	17.26	2.67
A. indica (KSA) leaves (Alq)	86.18	38.24	2.25
A. indica (Sudan) fruits (Afs)	25.6	21.84	1.17
A. indica (Sudan) leaves (Als)	115.8	18.43	6.28
A. mexicana whole plant (Am)	63.64	33.6	1.90
X. brasilicum whole plant (Xb)	15.72	7.19	2.19

KSA: Kingdom of Saudi Arabia; *A. indica: Azadirachta indica; A. Mexicana: Argemone Mexicana*; SI: Selectivity Index; *X. brasilicum: Xanthium brasilicum*

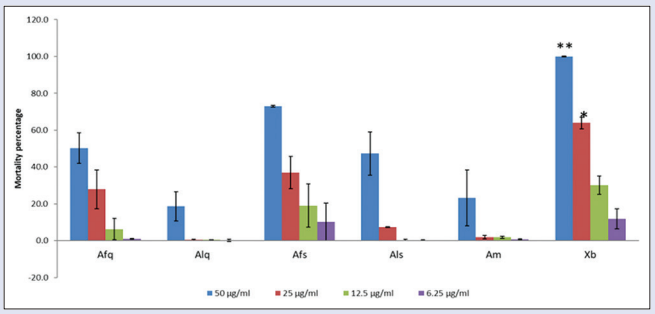

Figure 2: Mortality percentage of Vero cells with selected extracts. *$P < 0.05$, **$P < 0.01$

extracts tested exhibited different levels of antiparasitic activity against *T. gondii* at the highest concentration (50 µg/ml), ranging from 53.7 up to 100% parasitic inhibition, with all values above 50% and an IC_{50} in the range of 7.2–38.2 µg/ml. Many factors can cause variation in the activity level, such as the plant species, chemical composition, and part used.[33,34] In addition, different results can be obtained within the same plant and part, as in the case of *A. indica*, where the fruits and leaves collected from Al Rass, Qassim, KSA, and those obtained from Khartoum, Central Sudan, achieved different results due to soil type, climate, altitude, time and method of collection, and factors that have been previously mentioned.[35,36] Of all the plants examined, *X. brasilicum* whole plant methanol extracts proved to be the most potent, with an IC_{50} of 7.2 µg/ml and parasitic inhibition ranging from 52.4% to 100% for the 6.25 and 50 µg/ml concentrations, respectively. The methanol extract of *X. brasilicum* was found to have potent activity against different types of *Plasmodium* spp. *in vitro* and *in vivo*.[12,37] These findings are a good indicator of *X. brasilicum* methanol extracts as a potent agent against different apicomplexan protozoal species. Moreover, the plant methanol extract was also found to be effective against different types of flagellated protozoa, including *Trypanosoma rhodesiense, T. cruzi, L. donovani,* and *Trichomonas vaginalis*.[12,38] These results indicate that the plant methanol extracts act as general antiprotozoal agents. However, in a previous investigation of the antitoxoplasmal activity of an aqueous extract of *A. indica* leaves against *T. gondii* RH strain, a highly potent result was observed,[39] which contradicts our findings. It can be argued that there are many factors that can affect the biological activity of the plants, including different extraction solvents, sample regional locations, time of collection, and the age of plant species used in our current study.

Although the above-tested plant methanol extracts exhibited good antitoxoplasmal activity, all of the plants in our current study had differing levels of toxicity against the Vero cell line *in vitro,* ranging from 18.7% to 100% mortality, with a CC_{50} ranging from 15.7 to 115.8 µg/ml. Consequently, the SI was very low for all the extracts (<3.0) except for *A. indica* leaves obtained from Sudan (6.3). However, in previous research, many of the above plant extracts showed no toxicity *in vitro*,[40] possibly due to factors including type of extraction, type of cell, and other reasons, as discussed previously, affecting the biological activities. The results of atovaquone as the reference drug agree with the previously obtained results.[41]

CONCLUSION AND RECOMMENDATION

We can conclude that *X. brasilicum* whole plant methanol extract has potent antitoxoplasmal activity. We also recommend that all tested plant extracts should be subjected to phytochemical analysis for active ingredient isolation in addition to *in vitro* antitoxoplasmal activity identification, though this is not recommended for traditional uses due to the toxicity observed in this study.

Acknowledgements

The authors gratefully acknowledge Qassim University, represented by the Deanship of Scientific Research, for the material support of this research under the number (3879-alrasscac-2018-1-14-S) during the academic year 1439 AH/2018 AD.

Financial support and sponsorship

Deanship of Scientific Research, Qassim University, Saudi Arabia, supported this research as simple and continuous research project.

Conflicts of interest

There are no conflicts of interest.

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