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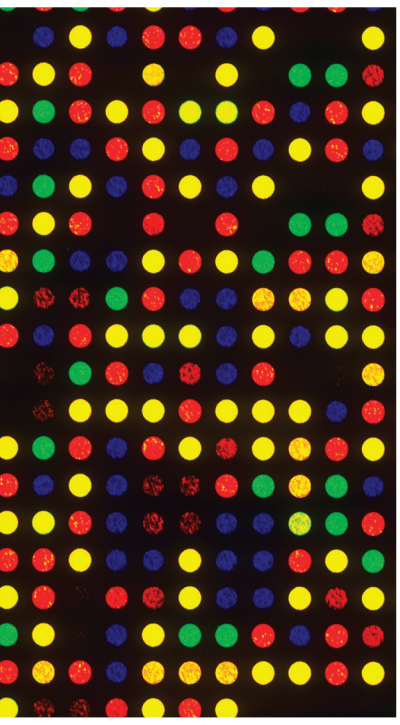
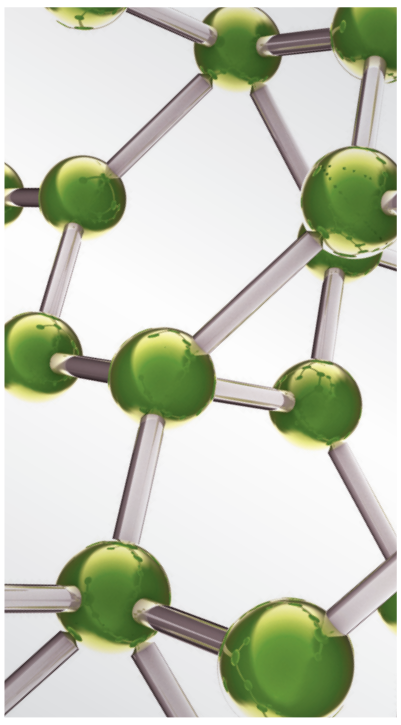
Guest Editors: John R. S. Tabuti, Imed Eddine Hassen, Umar Usman Pateh, and Mohamad Fawzi Mahomoodally



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Editorial Board

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Editorial

Recent Advances towards Validating Efficacy and Safety of African Traditional Medicines

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Traditional medicine (TM) has a significant role to play in the African health care system for two main reasons. The first one is the inadequate access to allopathic medicines and western forms of treatments. The majority of people in Africa cannot afford access to modern medical care either because it is too costly or because there are no medical service providers. Second, there is a lack of effective modern medical treatment for some ailments such as malaria or HIV/AIDS, which, although global in distribution, disproportionately affect Africa more than other areas in the world. It is generally believed that African TMs if revisited and meshed with recent developments in science could provide substantial benefits that could help give a boost to the African health care system and by doing so alleviate sufferings and livelihood of people. It is also believed that by rediscovering and adding value to the ancient wisdoms it will also empower people at the grass-root level and help patients as well as scientists find solutions to mitigate, *inter alia*, the global impact of diseases.

The last few decades have witnessed a growing number of documentations concerning materials used in traditional treatments in Africa. Nonetheless, there is still a paucity of clinical evidence to show that TMs are clinically effective and can be administered to patients safely. Without this information, users of TMs in Africa and elsewhere remain skeptical about the value of some therapies. Additionally, this denies people the freedom to choose complementary and alternative medicines that are potentially less costly and more accessible.

Another matter concerning TMs is the need to understand the clinical safety of these therapies. It is believed that the correct method of studying the effectiveness and potential of TMs should be solely based on scientific principles. If the remedies and practices applied by TMs are efficacious, they should be documented properly and their use should be supported and extended to other fields of practice. On the other hand, if they are found to be clinically ineffective or harmful, their use should be prohibited. One way to establish whether they are effective or not is to embark on robust randomized, double-blind controlled clinical studies. To this effect, there is an ever-pressing need to apply the principles of evidence-based medicine to African TMs, particularly herbal medicines, such that those therapeutic medicinal regimens which prove to be potent and safe become part of conventional medicine and those which fail to meet this standard become obsolete. For these reasons, updated information about efficacy and safety of TM is urgently required. Additionally, the documentation of medicinal uses of African plants and traditional systems is becoming

an urgent issue because of the rapid loss of the natural habitats of some of these plants due to anthropogenic activities and also due to an erosion of valuable traditional knowledge.

For the present special issue, original research and review articles that discuss aspects of efficacy and safety of African TMs have been considered. In this special issue, recent findings on evaluations of efficacies of TM from Africa for the management of obesity, stress, antioxidant activities, anticancer properties, and skin pigmentation are collated in 6 original research articles and an overview of priority medicinal plants and fungi in 3 key review articles.

The antiobesity effects of *Nitraria retusa* ethanol extract (NRE) in 3T3-L1 cells and in high fat diet-induced obesity in mice was studied by F. Z. Kalai et al. It was found that NRE administration significantly decreased body weight gain, fat pad weight, serum glucose, and lipid levels in high fat diet-induced obese mice. The mRNA expression results showed an enhancement of the expression of genes related to liver metabolism. It was suggested that NRE treatment had a protective or controlling effect against a high fat diet-induced obesity in C57B6J/L mice through the regulation of expression of genes involved in lipolysis and lipogenesis and thus the enhancement of the lipid metabolism in liver.

M. Ben Othman et al. investigated the antistress properties of *Cymbopogon schoenanthus* (CSEE), growing wild in the southern part of Tunisia and used in local folk medicine as diuretic, antispasmodic, rheumatism, anorexia, and digestive disorders including food poisoning. They demonstrated that pretreatment of SH-SY5Y cells with CSEE significantly inversed H_2O_2-induced neurotoxicity. Moreover, CSEE treatments significantly reversed heat shock protein expression in heat-stressed HSP47-transformed cells and mRNA expression of HSP27 and HSP90 in H_2O_2-treated SH-SY5Y. They concluded that the effect of CSEE on animal behavior was concordant with a significant regulation of blood serum corticosterone and cerebral cortex levels of catecholamine.

The efficacy of treatment of knee osteoarthritis using Shea nut oil (an extract from the indigenous African *Vitellaria paradoxa* tree) was validated by S.-p. Chen et al. The main findings showed significant change between the baseline and post-16-week testing, both in terms of morphological changes and muscle activity. Pain significantly reduced by the 16th week. Indeed, after sufficient dosage and intervention, the effects of Shea nut Oil include decreased inflammation, increased collagen, amelioration of pain, and improved muscle function. These results indicate that Shea nut oil extract can be used as a complementary option to improve the symptoms and function in relation to knee osteoarthritis.

M. Boulaaba et al. studied the phenolic contents, antioxidant activities, and anticancer effects of *Arthrocnemum indicum*, a traditional medicinal halophyte used in the treatment of poisonous snakebites and scorpion stings in Tunisia. The antiproliferative effect as compared to the control was characterized by substantial total polyphenol content and high antioxidant activity. DAPI staining revealed that these extracts decrease DNA synthesis and reduce the proliferation of Caco-2 cells which are stopped at the G_2/M phase. The changes in the cell cycle-associated proteins correlate with the changes in cell cycle distribution. Eight phenolic compounds were also identified. In conclusion, *A. indicum* was found to possess interesting antioxidant capacities associated with a significant anti-proliferative effect explained by a cell cycle blocking at the G_2/M phase.

The melanogenesis regulatory effect of argan oil (*Argania spinosa*) was evaluated by M. O. Villarel et al. using B16 murine melanoma cells. The oil treatment was reported to cause MITF phosphorylation which subsequently inhibited the transcription of melanogenic enzymes. The inhibitory effect of argan on melanin biosynthesis was attributed to tocopherols as well as the synergistic effect of its components. The results of this study tend to provide the scientific basis for the traditionally established benefits of argan oil and present its therapeutic potential against hyperpigmentation disorders.

M.-S. Wu et al. revealed the cytotoxic effect of the flavonoid fisetin (FIS) on human COLO205 colon cancer cells in the presence and absence of the HSP90 inhibitors, geldanamycin (GA), and radicicol (RAD). A reduction in p53 protein with increased ubiquitin-tagged proteins was observed in COLO205 cells treated with FIS. Furthermore, GA and RAD reduced the stability of the p53 protein in COLO205 cells under FIS stimulation. The evidence supports HSP90 inhibitors possibly sensitizing human colon cancer cells to FIS-induced apoptosis and treating colon cancer by combining HSP90 inhibitors with FIS deserves further *in vivo* study.

M. F. Mahomoodally has provided an updated overview of 10 promising medicinal plants from the African biodiversity which have short- as well as long-term potential to be developed as into phytopharmaceuticals to treat and/or manage a panoply of infectious and chronic conditions. On their part H. El Enshasy et al. have summarized currently available information related to the nutritional and medicinal value of African and Middle Eastern macrofungi and highlighted their application in complementary folk medicine in this part of the world. Lastly, S. Z. Moghadamtousi et al. reviewed information and findings concerning current knowledge on the biological activities, pharmacological properties, toxicity, and chemical constituents of the eastern Nigerian species—*Loranthus micranthus*, which has been widely used in Nigeria and South Africa as ethnomedicine for treatment of hypertension, diabetes, and schizophrenia and as an immune system booster.

Within the structure of enhancing the significance of African TMs, this special issue has highlighted several aspects related to the efficacy and safety of traditional African treatments, particularly medicinal plants, with a focus on promoting rational utilization for better health care. Much more research and documentation is still required to achieve this ambitious goal.

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Research Article

Validating Efficacy of Shea Nut Oil Extract in Knee Osteoarthritis Patients

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Objectives. To examine and investigate the efficacy of shea nut oil extract (SheaFlex75) in relation to knee osteoarthritis (OA). *Methods*. Thirty-three patients (age 63.6 ± 5.8 years) with knee OA were recruited. Real-time ultrasound imaging and surface electromyography were used to objectively assess the morphological changes and the activity of vastus medialis oblique (VMO) muscles during a 16-week intervention of SheaFlex75. The intraclass correlation coefficient (ICC) was calculated to examine the reliability of the interscans. A paired-sample t-test was used to compare the findings in different stages. The Spearman's rank correlation coefficient was used to examine the relationship between the relevant variables of OA and percentage of thickness change of VMO at different contraction levels. *Results*. The baseline findings showed strong correlation, suggesting that the reliability of interscans at pretest was high. The ability to contract the muscles of the knee to a 30% contraction level showed significant change between the baseline and after 16-week testing, both in terms of morphological changes and muscle activity. Pain scale reported a significant decrease at the 16th week. *Conclusion*. The results suggest that SheaFlex75 can relieve the symptoms of knee OA and can result in improvement of muscle control of the knee.

1. Introduction

Vitellaria paradoxa, commonly known as the "shea tree," is a tree of Sapotaceae family, indigenous to Africa. The shea fruit consists of a thin, tart, nutritious pulp, surrounding a relatively large, oil-rich seed, from which "shea butter" is extracted. The butter has been used locally as food, providing a major source of dietary fat. In the West, shea butter is most commonly used in cosmetics [1, 2]. Extracts from the seed have also been used for the treatment of arthritic conditions [3, 4].

Osteoarthritis (OA), also known as degenerative arthritis, degenerative joint disease, and osteoarthrosis, is a group of mechanical abnormalities involving degradation of joints, including articular cartilage and subchondral bone. OA is the most common form of arthritis [5] and the leading cause of chronic disability which affects about twenty-seven million people in the United States [6, 7] and nearly 8 million people in the United Kingdom [7].

It most commonly affects the knee and has an impact on the health-related quality of life of the elderly [5]. Symptoms may include joint pain, tenderness over the inside of knee, stiffness, locking, reduced mobility, atrophy of lower extremities, and decreased walking speed. These functional impairments may reduce a sufferer's general level of exercise and increase the risk of consequent injuries, such as those that might result from a fall.

Patients with knee OA demonstrate different kinematics and kinetics of gait pattern from the healthy adults. These changes may restrict the functional ability of the elderly in daily life, such as walking, stair climbing, navigating obstacles, and standing up. The muscle activation patterns of the key lower extremity muscles involved in the gait were reported to avoid pain and protect the knee from further

degeneration. These muscle activities were reported to be different between patients and healthy adults. A study by Stauffer et al. compared patients with knee OA and healthy young adults and reported less knee joint motion, isometric knee strength, and peak ground-reaction force in patients [8]. Suzuki and Takahama [9] and Benedetti et al. [10] observed increased activity of the quadriceps and hamstrings during weight bearing. Kaufman et al. compared the gait patterns during level walking between the patients group (n = 139, aged between 30 and 82 years old, grade II OA severity) and healthy controls (mean age = 30 years old) and also found less peak knee motion (54° versus 60°) in the knee OA cohort. The walking speed in the knee OA group was lower than the healthy control [11]. Childs et al. reported that muscle coactivation of the lower extremities showed significant increased and decreased knee excursion during walking [12].

The existing conservative intervention includes medication (such as nonsteroidal anti-inflammatory drugs and steroid joint injections), physical therapy, knee braces, and injections of hyaluronic acid. If disability is significant and the conservative managements are ineffective, surgical knee arthroplasty may be recommended [7]. Recently, another alternative supplement, shea nut oil extract, was proven to be effective. In 1998, US Food and Drug Administration approved shea nut oil as a safe food additive. The traditional Africans have used shea nut oil extracts to treat arthritis, but the mechanism has not been clear. Cheras and his colleagues carried out a 15-week random double-blind OA biomarkers study to compare the effectiveness of SheaFlex70 (a triterpene-rich extract of *Vitellaria paradoxa*) intervention in comparison to placebo groups. The OA biomarkers were found to be significantly decreased in comparison with the placebo group [3]. Experimental and clinical studies showed SheaFlex70 could effectively reduce cytokine, showing improved cartilage retention, bone retention, and pain management [3, 4]. Quadricep strength declines with age, with some evidence to suggest consequent functional impairment. O'Reilly et al. found that patients with symptomatic knee OA demonstrate incomplete activation of the quadriceps and also showed that quadriceps weakness was associated with impaired function. Whether shea nut oil extracts can assist to improve the function of quadriceps was not yet been proven [13].

The few studies that proposed a possible mechanism for the effects of shea nut oil extracts on knee OA have offered comments restricted to the improvement of symptoms or the inflammatory changes within blood. The influence of shea nut oil extracts on patients' functional activity and ability to control or modulate knee function has not yet been explored. This study attempts to do so, investigating the influence of shea nut oil extracts on these functional activities and neuromuscular control of knee OA.

2. Materials and Methods

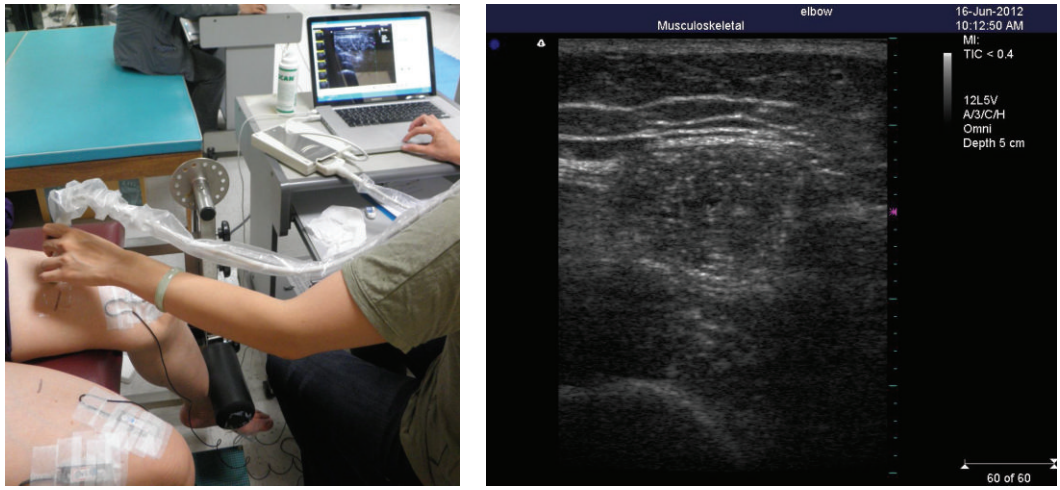
2.1. Subjects and Tasks. The group of subjects with OA consisted of volunteers (aged 63.6 ± 5.8 years), 10 males and 23 females, recruited from the outpatient department of the Physical Medicine and Rehabilitation Department, China Medical University Hospital, Taichung, Taiwan. Participants with knee OA had radiographic evidence of bilateral knee OA even if their symptoms were unilateral.

Subjects with knee OA were included if (1) they had been diagnosed with knee OA in line with 1986 American College of Rheumatology clinical criteria, (2) radiographic examination exceeded criteria greater than grade 2 of the Kellgren and Lawrence radiographic criteria (grades ranging from 0 to 4, with 0 being normal and 4 severe OA) [14], (3) they had inner-knee pain, (4) they had morning joint stiffness for over half hour, and (5) they had knee clicking during activities.

Subjects were excluded if they (1) were unable to independently walk or were walking with assistive devices, (2) had a neurological systemic diseases, such as Parkinson's disease, Alzheimer's disease, and Multiple Sclerosis (3) had traumatic injuries or fractures of a lower extremity (hip, knee, or ankle joints), (4) had rheumatoid arthritis or relevant arthritis such as metabolic arthritis (gout), (5) had any surgical intervention on a lower extremity such as arthroplasty, amputation, or ligament reconstruction, (6) had restricted range of motion of lower extremity joints (hip, knee, and ankle), (7) were to undergo physical therapy during the experimental period, or (8) were unable to understand the experimental protocol. All subjects signed an informed consent document approved by the China Medical University Hospital Institutional Review Board prior to taking part in this study.

2.2. Experimental Procedure. This is a nonrandomized control, intervention study. Ultrasonography and surface electromyography (s-EMG) were used in this study to provide the objective assessment of the changes to muscle and to investigate the influence of treatment with shea nut oil extract (SheaFlex75). Each participant was requested to attend three sessions to establish baseline conditions and then for assessment after 8 weeks and after 16 weeks. The intervention protocol was to take 6 pills per day for 16 weeks. The morphological changes of muscles around the knees and the ability to control muscles in different tasks were examined at each of the three sessions. The subjective findings were assessed by subjective pain intensity and modified Lequesne index to assess the pain and functional impairment. To avoid intraexaminer difference, the same examiner assessed the questionnaires and real-time ultrasound applications. The other two examiners performed the EMG applications and instruction.

2.3. Subjective Pain Intensity, Visual Analog Scales (VAS). Participants were requested to scale the subjective intensity of pain using the VAS. VAS is an assessment tool for patients to self-assess pain intensity. This tool is widely applied clinically to assess the improvement of pain in patients with musculoskeletal dysfunction. The questionnaire was composed of a 10 cm continuous line between two end-points. One end is 0 and the other end is 10. The "0" indicates no pain and the "10" indicates the most severe pain (intolerable pain). Each patient specifies his/her level of agreement to a statement by indicating a location along this line.



(a) (b)

Figure 1: The examiner used the linear transducer to capture the image of the vastus medial (VM) at each muscle status (a) and the scanned image (b).

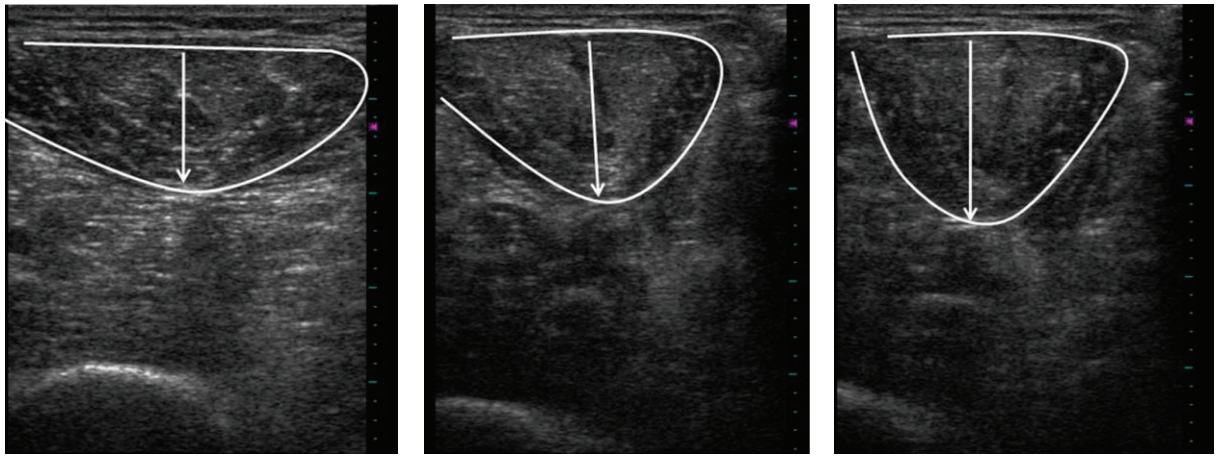
2.4. Modified Lequesne Index in Knee Osteoarthritis. The Modified Lequesne index in knee OA, which was developed in France in 1970 and was published in 1980, is an assessment tool for the functional characteristics of knee OA. Eleven questions are used to evaluate these characteristics, including knee pain, stiffness, walking, squatting, and stair climbing. The sores are between 0 and 24. Faucher et al. reported high reliability and validity of the questionnaire in the context of determining symptom changes and function assessment of knee OA patients [15].

2.5. Rehabilitative Ultrasound Imaging (RUSI). Physicians use ultrasound as a diagnostic imaging modality and also as "interventional ultrasound" or "invasive ultrasound" to assist and guide procedures during examination or surgery [16]. For the rehabilitation professional, ultrasonography is not a stand-alone assessment or treatment tool but is incorporated alongside existing clinical skills. In such a context, it is primarily deployed for viewing both static and dynamic muscle performance to investigate neuromuscular control and identify underlying dysfunction. These findings provide foundation information for assessment and treatment of neuromuscular control and the design of training regimes. As used by therapists, it is termed "rehabilitative ultrasound imaging" [17]. In general, RUSI provides a relatively low-cost and noninvasive way to examine the muscles, but its sensitivity for effective detection of muscle changes as an outcome measurement in an intervention study remains to be determined.

In this study, ultrasound images were taken using a linear transducer (>7.5 MHz). Images of the vastus medial (VM) were captured both at rest and in contracted states at two different knee positions (see Figure 1). All images were downloaded to a computer to be measured offline using Image "J software 1.4." During scanning of the knees, each participant was positioned in an erect sitting position, on a NK table using a device to standardize the position of knee. The site for scanning was at the distal height at one-third of the distance between anterior superior iliac spine and medial tibial plateau, in a standing posture. The asymptomatic or less symptomatic side was examined first, and each task was performed three times. Rest images were taken bilaterally at the position of knee flexion at 90 degrees. Subsequently, subjects were requested to activate knee extensors to the maximum by extending the knee against the pad at the same posture. The examiner then adjusted the torque arm of NK table to 60 degrees. The maximal voluntary effort (MVE) and 30% of MVE were performed at this position, separately. The same protocol was replicated for the other leg. As expected, muscle thickness increased with the level of effort. Since the actual thickness of muscle was different (see Figure 2), percentage changes were useful for comparing the general changes within the three sections. The equation is

$$\frac{(\text{Thickness at contraction status} - \text{Thickness at rest})}{\text{Thickness at rest}} \times 100\%. \quad (1)$$

2.6. Surface EMG Protocol. The EMG signals from 8 muscles of the bilateral vastus medial (VM), vastus lateralis (VL), medial hamstring (MH), and gastrocnemius (GM) were collected by the band-pass filter at 2–400 Hz and at a sample rate 1000 Hz (using Biopac Systems MP150 and EMG 100B, Biopac, USA). Following the SENIAM skin preparation protocols, the alcohol swabs were used to wipe skin and then the electrodes were placed. The electrodes were oriented on the centre of the muscle belly in a longitudinal fashion in the direction of the muscle fibers. Placement of the electrode was facilitated by palpating the muscle as the subject contracted the muscle against resistance (Figure 3). A reference electrode was placed over the radius styloid process of the right wrist.



(a) VM at rest (b) VM at MVE (c) VM at 30% of MVE

Figure 2: The scanned images of the vastus medial (VM) at three different muscle statuses. The overlay depicts the shape of VM and the distance between opposite points measured as the thickness of the VM.

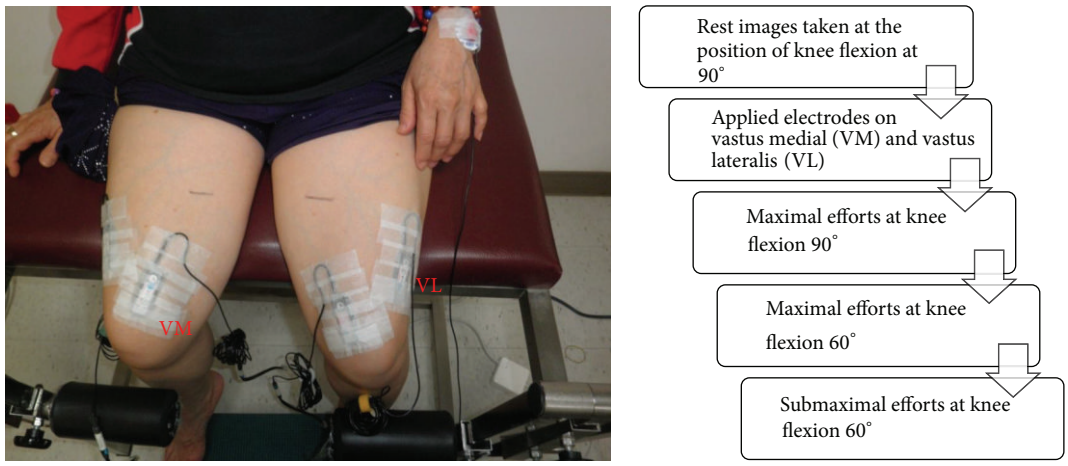


Figure 3: Examples of electrode positions for VM and VL placement. The ultrasound and s-EMG data is synchronously collected. The experimental protocol is listed, right.

Reusable electrodes were used to detect the signals from muscles.

The electrodes over the VM and VL were placed with the subject in a 90-90 sitting posture on the NK table, and then the MVIC of these two muscles collected with ultrasound synchronously (Figure 3). Each participant was requested to extend the tested leg against the pad of N-K table continuously for 5 seconds. Data for the MVIC and 30% MVIC of VM and VL were collected at 60 degrees of knees separately. The electrodes over the right and left hamstrings were placed with the subject in a prone position. Each participant was requested to flex the knee maximally against resistance from the examiner. The magnitudes of muscle forces were smoothed using a moving root-mean-square (RMS) filter with a 25 ms window. To facilitate comparison among sessions, the s-EMG was normalized to the mean of the RMS values obtained during three 3-seconds maximal voluntary isometric contractions of each of the muscles [18].

2.7. Data Analysis Strategy. The age, body mass, height, and body mass index (BMI) of the subjects are presented in the descriptive statistics. The analysis of ultrasound imaging data was conducted using the statistical methods shown below, and the descriptive findings of the measurements taken are then given. The intraclass correlation coefficient (ICC) was considered to be an appropriate statistical method for analysis of repeated measurements of muscle thicknesses [19–21] and was calculated to examine the reliability of the inter-scans. The Spearman's rank correlation coefficient was used to examine the relations among the variables of OA severity level, pain duration, pain scale, and percentage change in thickness of VMO at different states of contraction. A paired-sample t-test was used to assess the difference between the

Table 1: Demographic data of participants completing the three studies ($n = 33$).

	Mean	SD
Age (y/o)	63.6	5.8
BW (kg)	66.8	11.8
Height (m)	1.6	0.1
BMI (kg/m^2)	26.5	4.5

Table 2: Descriptive findings of the percentage change in thickness among rest and different contraction statuses in VM.

	Percentage change in thickness	
	Mean (%)	SD (%)
Rt_90_dif	19.5	14
Lt_90_dif	17.5	12.9
Rt_60_dif	16.5	11.8
Lt_60_dif	18.3	13.5
Rt_60_sub_dif	15.5	12.3
Lt_60_sub_dif	15.6	12.6

Rt: right; Lt: left; 90: MVC at knee flexion 90°; 60: MVC at knee flexion 60°; submax: submaximal effort (30% of MVC) at knee flexion 60°.

percentage change in thickness and muscle amplitude. A P value of less than 0.05 was taken to indicate significant difference.

3. Results

3.1. Descriptive Findings. In this study, 33 patients completed the three sections of the experimental protocol. The demographic data of the participants are listed in Table 1. The descriptive findings of the percentage change in thickness among rest and various contraction states of the VM are listed in Table 2.

3.2. Reliability of RUSI in OA Patients. Inter-scan reliability at the baseline secession was reported as moderate (ICC > 0.85) to approve the stability of skills, indicating acceptable levels of consistency of subject behavior during the trials. The Spearman's rank correlation coefficient was used to assess the correlations and the presence of other possible variables which may have been affecting the muscle contractions. The OA severity level, pain duration, and pain scale ($P > 0.05$) showed no significant difference with the percentage of muscle contractions. The pain duration was defined in three groups: group 1 as 6–12 months, group 2 as 1–5 years, and group 3 as more than 5 years (Table 3).

3.3. Comparisons of the Findings within the Three Trials. The paired-sample t-test was used to compare the findings at baseline, after 8 weeks, and before 16 weeks. Significant findings in relation to the percentage change in thickness at submaximal efforts between the baseline and after 16 weeks were reported ($P = 0.02$) (Table 4). The modified Lequesne index includes 4 sections; pain, stiffness, mobility, and total work. The paired-sample t-test was used to compare values acquired from subjects during the three sessions. The pain section and stiffness section showed a significant difference, with $P = 0.01$ and $P = 0.04$, respectively (Table 4). The VAS pain was reported as significantly different ($P = 0.03$) between the baseline and after 16 weeks sessions, showing declined signs as the intervention progressed (Figure 4). The average amplitude of VM and thickness change of VM were compared among the three sessions as well, and significant findings were reported between the baseline and after 16-week stages ($P = 0.04$) (Table 4).

Table 3: The correlation between muscle thickness change and other possible contributary factors.

Baseline data	Correlation coefficient	P value
OA severity level		
PTC at 90	−0.2	0.15
PTC at 60	−0.09	0.51
PTC at submax	−0.02	0.88
Pain duration		
PTC at 90	0.03	0.85
PTC at 60	−0.11	0.42
PTC at submax	−0.15	0.26
Pain scale		
PTC at 90	0.06	0.69
PTC at 60	0.05	0.73
PTC at submax	0.05	0.74

PTC: percentage of thickness change; 90: MVC at knee flexion 90°; 60: MVC at knee flexion 60°; submax: submaximal effort (30% of MVC) at knee flexion 60°.

Table 4: Comparison of the findings at baseline, after 8 weeks and after 16 weeks.

Pair t-test	Baseline—after 8 weeks (P value)	Baseline—after 16 weeks (P value)
RUSI		
90	0.11	0.81
60	0.34	0.97
60 submax	0.73	0.02*
EMG		
90	0.24	0.12
60	0.6	0.32
60 submax	0.08	0.04*
Questionnaires		
Pain	0.03*	0.01*
Stiffness	0.07	0.04*
Mobility	0.17	0.08
Total work	0.57	0.25

*$P < 0.05$, significance.
90: MVC at knee flexion 90°; 60: MVC at knee flexion 60°; submax: submaximal effort (30% of MVC) at knee flexion 60°.

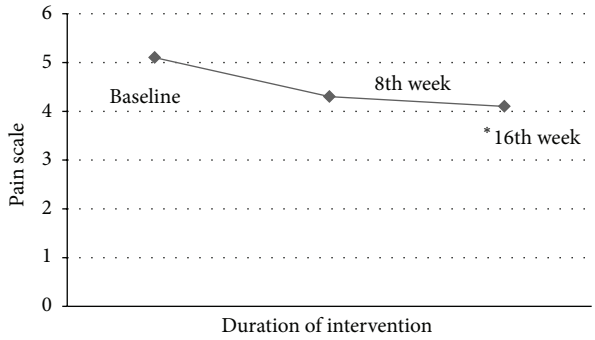


Figure 4: Symptom Change of VAS during the three trials (∗ indicates significant difference in comparison with the baseline).

4. Discussion

4.1. Summary of Important Findings in This Study. The symptom relief and improved muscle control were observed both in ultrasound and s-EMG findings at sub-maximal effort after the 16-week intervention. This indicates that shea nut oil extract can significantly relieve the symptoms in question and the subject's response to muscle activity and further the morphological changes after 16-week intervention.

4.2. The Relation between Muscle Thickness and Muscle Activity. Changes in muscle thickness from the resting state to a contracted state are considered to reflect changes in muscle activity level, although the linearity of the relationship is controversial [22, 23]. Although muscle thickness change and amplitude of EMG are likely to be a positively correlated, the relationship is certainly not simple enough to be linear and perhaps only marginally curvilinear over a small range. As such, the relationship between muscle thickness and EMG remains inconclusive [24–26]. Strasser and his colleagues [27] carried out a randomized and observer blind study to investigate whether the quadriceps measured by real-time ultrasound correlated with isometric maximum voluntary contraction force (MVC) of quadriceps in young and old sarcopenia. They found that thickness of VM had the best correlation with MVC in the elderly and showed that measurement of muscle thickness, especially of VM by real-time ultrasound, is a reliable method for monitoring the extension of quadriceps. These clinical data support our finding that thickness measurement by real-time ultrasound could be a reliable and valid technique as an objective assessment to monitor muscle function. As the significant morphological changes and muscle amplitudes only occurred at the sub-maximal effort, it can be suggested that the ability to voluntarily control or adjust the strength has been improved, while no claim relating to increased capability at maximal effort can be made.

4.3. The Possible Analgesic Effect of the Shea Nut Oil Extract. Postexercise induced muscle soreness (PEMS) is a dull, aching pain combined with tenderness and stiffness [28], usually following unaccustomed eccentric exercise [29, 30]. Maclntyre and his colleagues found that neutrophil levels were greater in the exercised muscle than in nonexercised muscle and the delayed onset muscle soreness was increased from 0 to 48 hours, and eccentric torque decreased from 2 to 24 hours. Significant relationships were found between interleukin 6 (IL-6) levels at 2 hours and delayed onset muscle soreness at 24 hours after exercise. These findings suggest a relationship between damaged to the contractile proteins and inflammation. A significant relationship of the severity of PEMS after eccentric exercise and IL-6 has been shown. Intense eccentric exercise results in high tension and mechanical damage in the muscles that is followed by increased amounts of IL-6 release, which corresponds to the time-course development of muscle soreness [31]. Arendt-Nielsen and his colleagues [32] conducted a randomized, prospective, double blind, placebo-controlled, parallel group study demonstrating that prophylactic supplementation by shea nut oil extract significantly relieves muscle tenderness associated with intensive eccentric exercise, most likely via reduction of proinflammatory cytokine IL-6.

In the present study, clinical data showed that the knee OA symptoms, in particular pain and stiffness, improved significantly after the 16-week intervention. The preliminary results have proven an analgesic effect of the shea nut oil extract that is consistent with the currently existing studies [3, 4]. Pain reduces strength and endurance of the muscles by inhibiting muscle recruitment and thus impeding motor control [33, 34]. Muscle contraction should be improved and patients may regain part of their motor control ability.

4.4. The Possible Biologic Mechanisms of the Shea Nut Oil Extract in Knee OA. The study by Alander and Andersson [35] concluded that fractionated shea butter caused a significant reduction of the inflammatory response of human keratinocytes in comparison to croton oil. The active agent of shea tree extracts contains triterpenes, derived from the seed of the shea tree, *Vitellaria paradoxa*. The most abundant triterpenes are butyrospermol, lupeol, and the α and β-amyrin, in addition to their dihydroderivatives. Findings that the triterpenes have an anti-inflammatory effect and can suppress NF-κB activation were noted in previous studies [36, 37].

Cheras et al. conducted a randomized double-blind placebo-controlled trial to investigate the potential modes of action of the triterpene-rich shea tree nut extract, in the treatment of osteoarthritis. They concluded that treatment with shea nut oil extract during the 15-week duration of the study showed that subjects with elevated biomarkers presented a range of anti-inflammatory and chondroprotective effects together with a potential for beneficial modulation of bone formation. Significant reductions in inflammation were shown by decreased TNF-alpha, hsCRP, and IL-6, which all fell by over 20% in the treatment group and the reduction in C-telopeptide fragments of type II collagen (CTX-II) over the 15-week study across the entire active group and in the active subgroup with high levels at baseline. This is consistent with chondroprotective activity of shea nut oil extract [3]. Their findings that inflammation was decreased over 15 weeks are consistent with the current results that the symptoms and

muscle functions are improved significantly after a 16-week intervention.

4.5. The Possible Influences of SheaFlex75 on Knee OA. Hodges and Moseley reviewed and summarized a number of mechanisms that have been proposed to explain the effect of pain on motor control in the spine and extremities. The majority of available hypotheses is broadly consistent with two main theories: the pain-spasm-pain model and the pain adaption model. The pain-spasm-pain model was suggested to be too simplistic, and so the current experimental and clinical data offer support for the pain adaption model [33]. Another review by Sterling et al. reported that clinical and basic science investigations have provided evidence in relation to changes of motor function under the effects of pain, including both increases and decreases in muscle activity, along with alterations in neuronal control mechanisms, proprioception, and local muscle morphology. A new model (the neuromuscular activation model) was used to explain how the patterns of muscle activation and recruitment are altered in the presence of both acute and chronic pain. The alterations seem to particularly affect the ability of muscles to perform synergistic functions related to maintaining joint stability and control [34]. These discussions address the notion that pain causes motor control changes, ranging from changes in recruitment to reduced strength and endurance of the muscles, and that the possible patterns are hyperactivity and hypoactivity.

Cheras et al. found that shea nut oil extract treatment over the 15 weeks of their study was effective in decreasing inflammation responses and relevant symptoms [3]. Knee OA is a chronic disease, and muscle function and bony structure progressively deteriorate over time, altering muscle activation and strength of key lower extremity muscles during weight-bearing activity. Quadricep muscle strengthening is a common management of knee OA in that it can delay OA progression. The ability to control and adjust the function of the quadriceps muscles may thus assist the treatment of knee OA. According to existing studies, the thickness change in real-time ultrasound and s-EMG activity of the back and VM muscles correlates linearly, showing that the greater the thickness change, the greater the amplitude of muscle activation [22–27].

In this study, ultrasonography is used primarily for viewing both static and dynamic muscle performance, to define neuromuscular control of normal muscles and those that present specific alteration caused by underlying dysfunction. The effectiveness of shea nut oil extract on the morphological changes of muscles in images is not clear. Herein, the present findings indicate that the contractile function of the knee extensors during sub-maximal effort was significantly changed after 16-week stage of intervention, compared with the baseline finding, but no significant difference was found between the baseline and after 8-week intervention. Maximum effort of the knee muscles was not applied during functional activities, and sub-maximal effort was used to control and coordinate the agonist and antagonist muscles to achieve the goal.

How changes in control relate to the fear associated with pain is still in question. It is proposed that fear of causing pain is critical in behavioral and motor output, and the fear-avoidance model is gaining considerable support in the literature [34]. The fear avoidance model argues that fear of pain and (re)injury prevents the normal return to activity, leading to deconditioning and disability, as reported by patients with lower back pain [33]. Patients with OA may behave similarly. If so, it seems reasonable that if deconditioned strength and endurance may not be regained by simple pain alleviation, they may be obtained through progressive engagement with exercise. As for the subjective functional assessment, patients' mobility and total symptoms were not reported as significantly improved. The findings indicated that improvement in symptoms did not immediately manifest in the activities of daily living, perhaps due to habitual compensative strategies. Our studies found that the ability to control and coordinate the contractile components was improved in the sub-maximal effort of muscles, but not in the maximal voluntary effort. It can be concluded that SheaFlex75 can alleviate pain and improve the contractile ability of knee muscles in knee OA after 16-week intervention.

4.6. The Limitation of This Study. Inevitably, difficulties were encountered during the study. It was designed to be a case-control intervention to investigate the validity of a proposed effect of shea nut oil extract on knee OA. Due to the difficulties in the recruitment of patients and commitment to the 16-week intervention, the study design did not include a placebo group. In addition, knee OA is a chronic and progressive disease; it was recognized that some patients may drop out for personal reasons or because they wish to accept another medical intervention such as physiotherapy or acupuncture to relieve their symptoms.

The morphological changes and muscle activity of VMO was used as the main outcome measurement. However, it is recognized that the possibility that of muscle imbalance may influence the ratio of the change in thickness and amplitude change in the quadriceps. The VL data was not collected as completely as that for the VM, although Strasser et al. reported a strong correlation between the strength and thickness of the VM in old sarcopenia [27]. That these findings can be applied to and properly support the notion of this study is not proven, due to the different experimental protocol and population.

A further complicating feature of the trials was that patients were observed to use compensative strategies to achieve the task set. It is suggested that this could be the result of fear of resulting pain or of habitual strategies and may have influenced observed muscle control.

As for the issue of thickness measurement, the longitudinal distance between muscle borders of the VM was calculated at the rest and during contraction. However, as the VM is an oval-shaped muscle in the transversal view, the ratio of cross-section area of the VM may be a more precise measurement. The individual muscle sizes varied, and some images were not within the range of the scans. Therefore, the measurement of longitudinal distance of VMO

was made. These limitations and practical issues may provide opportunities for future improvement.

5. Conclusions

The effectiveness of treatment of knee OA using shea nut oil, an extract from the indigenous African *Vitellaria paradoxa* tree, is proven. After sufficient dosage and intervention, its effects include decreased inflammation, increased collagen, amelioration of pain, and improved muscle function. Although improved muscle function was observed, including greater control and an increase in muscle strength to achieve a functional goal, the subjective feeling of improvement in the activities of daily living was not significant.

The findings have proven the efficacy of shea nut oil extract as a complementary option to improve the symptoms and function in relation to knee OA.

Authors' Contribution

Kang-Ming Chang had provided the same effort as Li-Wei Chou.

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Research Article

Antiobesity Effects of an Edible Halophyte *Nitraria retusa* Forssk in 3T3-L1 Preadipocyte Differentiation and in C57B6J/L Mice Fed a High Fat Diet-Induced Obesity

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Nitraria retusa is an edible halophyte, used in Tunisia for several traditional medicine purposes. The present study investigated the antiobesity effects of *Nitraria retusa* ethanol extract (NRE) in 3T3-L1 cells using different doses and in high-fat diet-induced obesity in mice. Male C57B6J/L mice were separately fed a normal diet (ND) or a high-fat diet (HFD) and daily administrated with NRE (50, 100 mg/kg) or one for 2 days with Naringenin (10 mg/kg). NRE administration significantly decreased body weight gain, fat pad weight, serum glucose, and lipid levels in HFD-induced obese mice. To elucidate the mechanism of action of NRE, the expression of genes involved in lipid and carbohydrate metabolism were measured in liver. Results showed that mice treated with NRE demonstrated a significant decrease in cumulative body weight and fat pad weight, a significant lowering in glucose and triglycerides serum levels, and an increase in the HDL-cholesterol serum level. Moreover mRNA expression results showed an enhancement of the expression of genes related to liver metabolism. Our findings suggest that NRE treatment had a protective or controlling effect against a high fat diet-induced obesity in C57B6J/L mice through the regulation of expression of genes involved in lipolysis and lipogenesis and thus the enhancement of the lipid metabolism in liver.

1. Introduction

Obesity is a rapidly growing epidemic worldwide, presenting an increase in the risk of morbidity and mortality in many countries across the world [1]. It has become an increasingly prevalent public health problem and represents the complex interaction of genetic, developmental, behavioral, and environmental influences [2]. The World Health Organization (WHO) defines obesity as an abnormal excessive fat accumulation detrimental to human health. Obesity has also been defined as an increased adipose tissue mass, which is the result of an enlargement in fat cells and/or an increase in their number [3]. Moreover, obesity is fundamentally a problem of energy balance in that self-evidently it can develop when energy intake exceeds energy expenditure, resulting in fat accumulation and excessive adipose tissue mass. Adipose tissue, in addition to its function as the major storage depot for triglycerides, is an active endocrine tissue sensing metabolic signals and secreting hormones called adipocytokines that affect whole-body energy homeostasis [4]. Since it is an endocrine organ, it has a fundamental role in metabolism and homeostasis regulation, where numerous chemical messengers called adipokines are released for better communication. The production and secretion of an excess or insufficient amount of adipokines may provide a molecular link between increased adiposity and the development of diabetes mellitus, metabolic syndrome, and cardiovascular diseases [5]. The main metabolic fuels of the body are glucose, fatty acids,

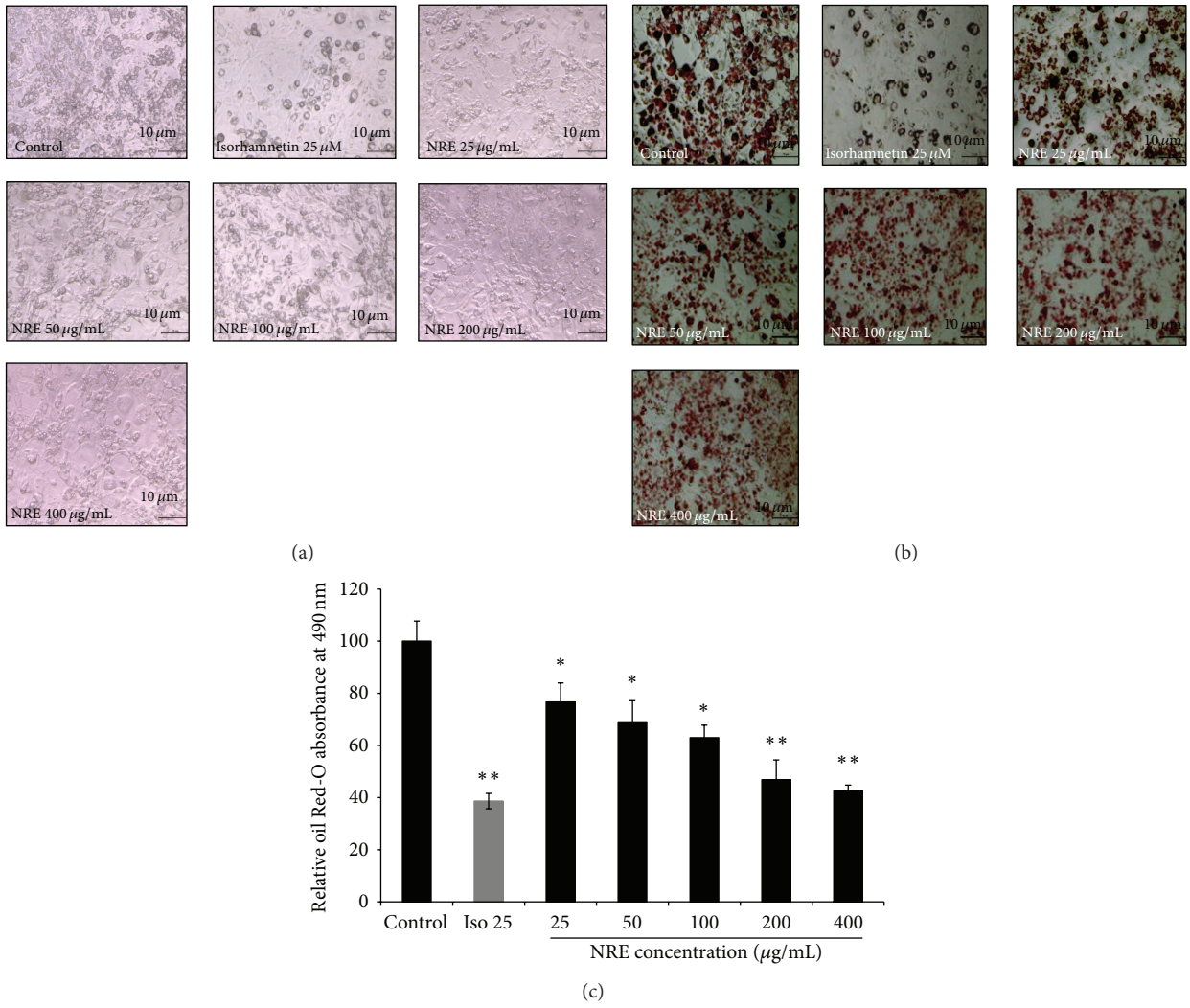

Figure 1: Effects of *Nitraria retusa* (NR) extract using different concentrations (25, 50, 100, 200, and 400 μg/mL) on cell differentiation and fat droplet formation in 3T3-L1 cells before (a) and after (b) oil Red-O staining. Fat droplets in preadipocytes and adipocytes differentiated for 9 days with or without *Nitraria retusa* (NR) and isorhamnetin (the positive control) treatments were stained with oil Red-O dye and examined using a light microscope. (c) Effects of *Nitraria retusa* extract NR on lipid droplet content in 3T3-L1 cells. Lipid droplet accumulation in treated cells was expressed as a percentage of control (untreated cells). Bars represent mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.005$.

and ketone bodies. In the metabolic homeostasis of the body as a whole, the liver occupies a central position. Indeed, besides building up glycogen in its own cells, the liver plays an essential role in the synthesis of adipose tissue triglycerides, by producing very low-density lipoproteins. Furthermore, the liver furnishes oxidizable substrates, not only to meet its own needs, but also to cover those of other tissues [6]. Besides, it has been reported that the liver plays an important role in modulating western diet-associated metabolic disorders. High-fat diets significantly alter the expression of many genes related to lipid, cholesterol, and oxidoreductive metabolism [7]. Nowadays, diets high in fats tend to promote obesity; hence inhibition of digestion and absorption of dietary fats are a biological remedy in treating obesity [8]. As synthetic drugs fail to give desired effects and with side effects involved, the utilization of traditional and alternative medicines is fast gaining acceptance. Medicinal plants are believed to harbor potential antiobesity agents that can act through various mechanisms either by preventing weight gain or promoting weight loss amongst them and this may be an excellent alternative strategy for developing future effective, safe antiobesity drugs [9]. Thus, the clinical importance of herbal drugs and polyphenols for treatment of obesity has received considerable attention [3]. These therapies have been variably efficacious on adipocyte differentiation and lipid accumulation in adipocytes. A number of herbal and dietary inhibitors of adipose differentiation have been identified, including isorhamnetin [10], Epigallocatechin-Gallate [11], quercetin [12], and Naringenin [13]. This family of phenolic compounds are potent scavengers of free radicals and potentially useful in the prevention of cancer and arteriosclerosis and also have been associated with several health promoting

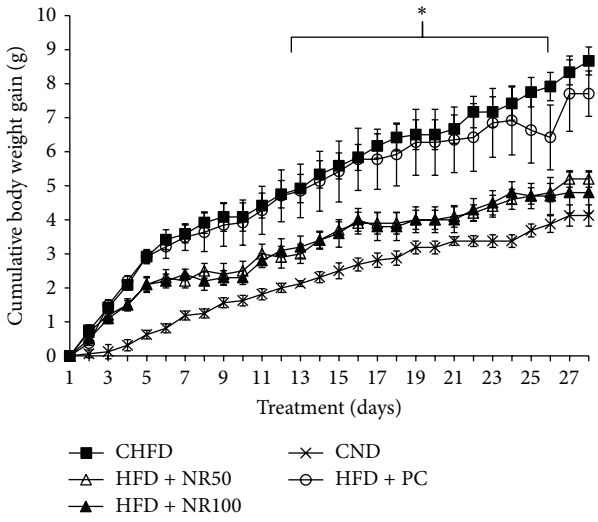

Figure 2: Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on cumulative body weight gain of mice fed with high-fat diet. Mice were fed normal diet (CND), high-fat diet (CHFD), high-fat diet supplemented with positive control Naringenin 10 mg/kg body weight (HFD + PC), high-fat diet supplemented with *Nitraria retusa* 50 mg/kg body weight (HFD + NR50), or high-fat diet supplemented with *Nitraria retusa* 100 mg/kg body weight (HFD + NR100) for 4 weeks. Body weight was daily measured at regular time. Data represent mean ± SEM, $n = 8$, $^{*}P < 0.05$, $^{**}P < 0.005$ compared to the high-fat diet group.

activities such as decreasing blood sugar levels and reducing body weight [14]. Halophytes are a salt-tolerant species from salt and arid regions and desert that can tolerate a wide range of environmental conditions and resist abiotic stresses such as salt, high temperature and luminosity, and drought stresses [15]. In fact, able to withstand and quench these severe environmental stresses, halophytes are equipped with powerful antioxidant systems that constitute mainly on phenolic compounds so-called "stress metabolites." These plants have ethnopharmacological data indicating their utilization in folk medicine. Thus, the role of these medicinal species in the prevention or treatment of diseases has been largely attributed to their antioxidant properties associated with a wide range of bioactive molecules [16]. *Nitraria retusa* is one of the native perennial halophyte species that belong to the botanical family Nitrariaceae. It is distributed in North Africa and restricted to Algeria and Tunisia. In Tunisia, it is widespread in central and south parts. This salt-tolerant and drought resistant shrub grows along shallow and hummocks on saline grounds near the coastal areas and produces fleshy red fruits from which a tasty and refreshing juice may be extracted. *Nitraria retusa* is known in Tunisia as "Ghardaq." The sweet drupes are edible for the treatment of hypertension. Leaves infusion and decoction are used as tea or cataplasm for their anti-inflammatory properties [17]. In previous chemosystematic investigation, the flavonoids contained in *Nitraria retusa* leaves were studied; six isorhamnetin glycosides (isorhamnetin 3-robinobioside, isorhamnetin 3-rutinoside, isorhamnetin-3-O-galactoside, isorhamnetin-3-o-glucoside, isorhamnetin 3-xylosylrobinobioside, and isorhamnetin-3-O-4Rhamgalactosylrobinobioside) and free isorhamnetin were identified [18]. These bioactive molecules isolated from *Nitraria retusa* have been reported to promote apoptosis in human myelogenous erythroleukemia cells [19] and to exert antioxidant and antimutagenic activities [20]. To the best of our knowledge, this is the first time to report the effect of *Nitraria retusa* ethanol extract (NRE) on inhibiting preadipocyte differentiation and lipid droplet accumulation in 3T3-L1 cells and reducing body weight in mice fed with high-fat diet in correlation with lipid metabolism in liver.

2. Materials and Methods

2.1. Plant Sampling. *Nitraria retusa* shoots were collected during August 2010 from the salt flat "Sabkha El Kelbia" located at N 35 48 44, E 10 09 06 (Kairouan, Tunisia). This locality is characterized by a semiarid climate with less rainfall <200 mm/year and higher salinity mean (20 g/L). The collected samples were rinsed with distilled water, kept in laboratory temperature, oven dried at 60°C, and then ground finely using a ball mill type "Dangoumeau." The plant powder obtained was stored at room temperature for further experiments.

2.2. Extraction Methods. Seventy percent ethanol extraction *Nitraria retusa* sample was conducted with 10% (w/v). The ethanol extract was kept in the dark at room temperature for 2 weeks, with shaking at least once a day. The liquid fraction was then collected, filtered through 0.22 μm filter (MILLIPORE, U.S.A.), and concentrated using SpeedVac (SCRUM Inc., Japan). The dried residue was redissolved in seventy percent ethanol or milli-Q water by vortexing and stored at −80°C for further experiments.

2.3. Cell Culture. Murine 3T3-L1 preadipocytes (Riken Tsukuba, Japan) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (5000 μg/mL)-streptomycin (5000 IU/mL) in 75 cm^2 tissue culture flasks. Medium was changed every 3 days and cell passage was carried out at 80% confluence at one on two ratio using 0.25% trypsin (1 mM EDTA). 3T3-L1 cells were cultured in a humidified incubator at 37°C and 5% CO_2.

2.4. Preadipocytes Differentiation and Oil Red-O Staining Procedures. 3T3-L1 preadipocytes were seeded into 96-well plates at 1.0×10^4 cell/well and cultured for additional two days until full confluence. Two days later (day 0), cells were incubated with a differentiation cocktail (MDI) containing 1/10 insulin solution, 1/10 dexamethasone solution, and 1/10 3-isobutyl-1-methylxanthine solution in standard culture medium for 3 days followed by additional 48 h with standard culture medium containing insulin alone. The differentiation-maintenance medium was changed every 2 days. To investigate the effect of *Nitraria retusa* on adipogenesis in 3T3-L1, NRE (25, 50, 100, 200, and 400 μg/mL) was

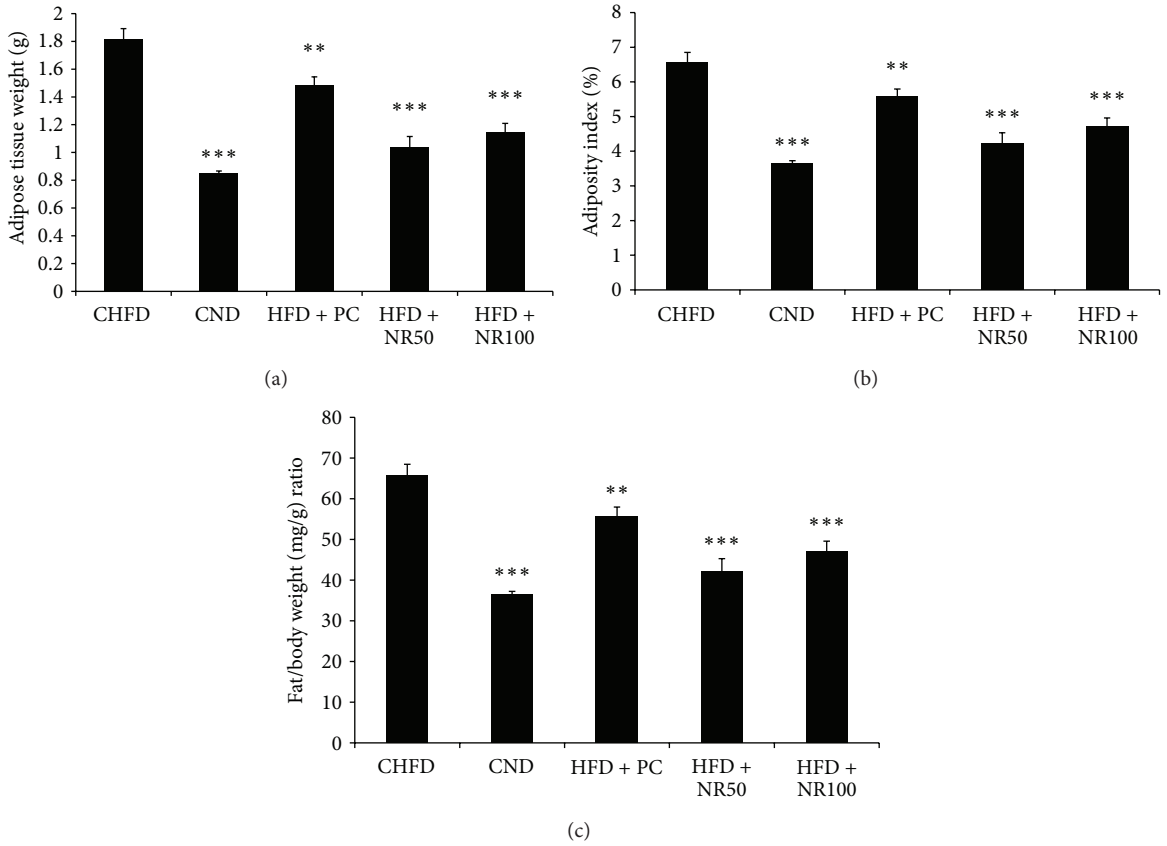

Figure 3: Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on adipose tissue weight (a), adiposity index (b), and fat/body weight (mg/g) ratio (c) of mice fed with high-fat diet. Mice were fed normal diet (CND), high-fat diet (CHFD), high-fat diet supplemented with positive control Naringenin 10 mg/kg body weight (HFD + PC), high-fat diet supplemented with *Nitraria retusa* 50 mg/kg body weight (HFD + NR50), or high-fat diet supplemented with *Nitraria retusa* 100 mg/kg body weight (HFD + NR100) for 4 weeks. At the end of experiment, adipose tissue, for all groups, was weighed. Data represent mean ± SEM, $n = 8$, $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ compared to the high-fat diet group.

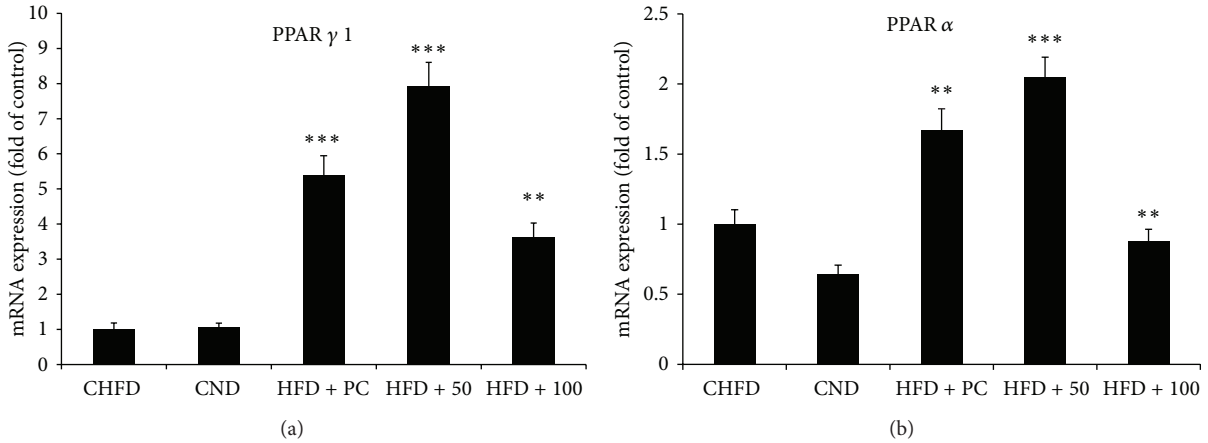

Figure 4: Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on genes regulating lipid metabolism in liver of mice fed with high-fat diet. Peroxisome proliferator activated receptor gamma (PPAR γ 1) (a) and peroxisome proliferator activated receptor alpha (PPAR α) (b). Real-time PCR was conducted and result was expressed as mRNA expression fold change compared to the control high-fat diet (HFD). Bars represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$ compared to the high-fat diet group.

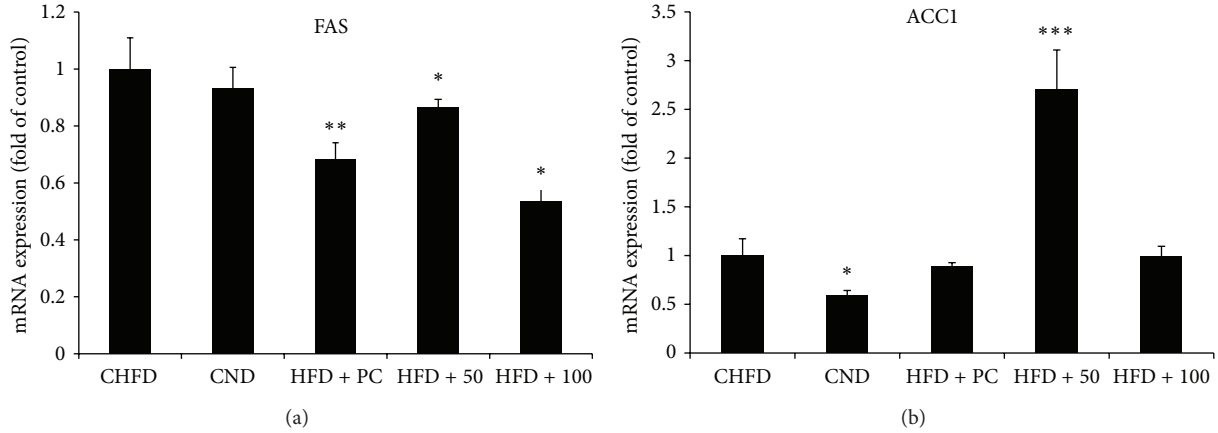

Figure 5: Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on genes regulating lipid metabolism in liver of mice fed with high-fat diet. Lipogenic enzymes; fatty acid synthase (FAS) (a) and Acetyl-CoA Carboxylase 1 (ACC1) (b). Real-time PCR was conducted and result was expressed as mRNA expression fold change compared to the control high-fat diet (HFD). Bars represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$ compared to the high-fat diet group.

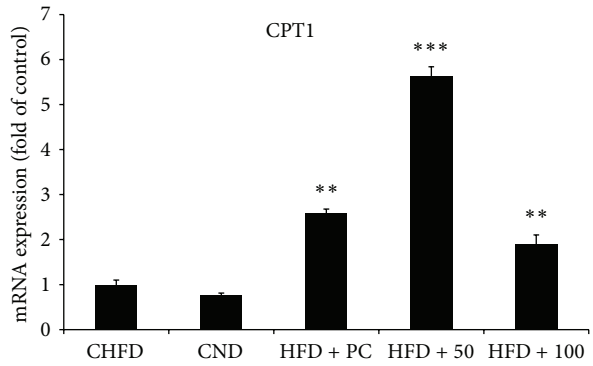

Figure 6: Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on genes regulating lipid metabolism in liver of mice fed with high-fat diet. Carnitine palmitoyltransferase I (CPT1) essential step in the beta-oxidation of long chain fatty acids. Real-time PCR was conducted and result was expressed as mRNA expression fold change compared to the control high-fat diet (HFD). Bars represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$ compared to the high-fat diet group.

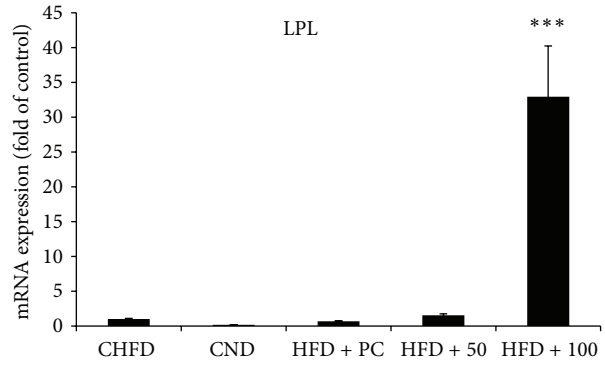

Figure 7: Effect of daily oral administration of *Nitraria retusa* (50 and 100 mg/kg body weight) on genes regulating lipid metabolism in liver of mice fed with high-fat diet. Lipoprotein lipase (LPL) enzyme responsible for the hydrolysis of triglycerides in lipoproteins. Real-time PCR was conducted and result was expressed as mRNA expression fold change compared to the control high-fat diet (HFD). Bars represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.005$ compared to the high-fat diet group.

added to the differentiation-induction and differentiation-maintenance media. The staining procedure was conducted according to the adipogenesis assay kit (Cayman Chemical Company). The absorbance was read at 490 nm with a 96-well plate reader. The lipid droplet content was reported as percentage of control cells, and isorhamnetin-treated cells were used as positive control.

2.5. Animals and Experimental Design. Four-week-old, male C57B6/JL mice were purchased from Charles River (Japan) and were maintained under a light cycle (12 h light/dark) and fed with a high-fat diet (HFD) or a normal diet (ND), purchased from Oriental Yeast Company (Japan) and according to the composition described in Table 1. After 1 week acclimatization, mice were divided randomly into 5 groups with 8 individuals for each group: control normal diet group (CND) fed with normal diet and orally administrated with water as vehicle, control high-fat diet group fed with HFD and orally administrated with water as vehicle (CHFD), high-fat diet group fed with HFD and orally administrated with Naringenin as positive control at a dose of 10 mg/kg of body weight (HFD + PC), high-fat diet group fed with HFD and orally administrated with *Nitraria retusa* at 50 mg/kg of body weight (HFD + NR50), and high-fat diet group fed with HFD and orally administrated with NRE at 100 mg/kg of body weight (HFD + NR100). Body weight and food intakes were measured daily at regular intervals during the feeding period (28 days). Following 4 weeks treatment, animals were sacrificed. Blood samples were collected and liver and fat tissue were dissected, weighed, and kept at −80°C until use. All procedures were performed in accordance with the Ethics Animal Care and Use Committee of the University of Tsukuba, Japan.

Table 1: Composition of experimental diets.

Ingredient (%)	Normal diet (ND)	High fat diet (HFD)
Casein	18.5	25.6
L-Cystine	0.28	0.36
Maltodextrin	29.01	6.01
α-Corn starch	9.67	16.00
Sucrose	2.00	5.50
Soybean oil	25.00	2.00
Lard	2.00	33.00
Cellulose	6.61	6.61
Mineral mix AIN 93G	3.50	3.50
Calcium carbonate	0.18	0.18
Vitamin mix AIN93	1.00	1.00
Choline bitartrate	0.25	0.25
Total	100	100

2.6. Biochemical Analysis of Blood Serum Analysis. Collected blood samples were centrifuged at 3,000 rpm for 15 min at 4°C. Then several metabolites like serum glucose, triglyceride (TG), total cholesterol (TCHO), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) levels were measured according to the kit manufacturer's instruction. The cytokine tumor necrosis factor-alpha (TNF-α) level in serum was also analyzed using the enzyme linked immunosorbent assay (ELISA) (Invitrogen Ms TNF-α kit) according to the manufacturer's instructions.

2.7. RNA Isolation from Liver and Real-Time PCR Analysis. 50 mg liver samples were homogenized using Polytron PT 1200 E homogenizer (Switzerland). Then total RNA was purified using the ISOGEN kit (Nippon GeneCo. Ltd., Japan) following the manufacturer's instructions. Total RNA was quantified and quality-checked using Thermo Scientific NanoDrop 2000 (USA) and the reverse transcription reactions were performed using the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad/CA, USA) using 1 μg of total RNA. Briefly, RNA was denatured by incubation at 65°C for 5 min, with 1 μL oligo (dT) primers, and chilled at 4°C. Then SuperScript III reverse transcriptase was added and the reaction mix was then incubated at 42°C for 60 min and then 10 min at 70°C [21]. The gene expression of peroxisome proliferator-activated receptor gamma 1 (PPAR γ 1), peroxisome proliferator-activated receptor alpha (PPAR α), lipoprotein lipase (LPL), fatty acid synthase (FAS), Acetyl-CoA Carboxylase 1 (ACC1), and carnitine palmitoyl transferase I (CPT1) were determined by real-time PCR, normalized to beta-actin, and reported as fold of control. Primers and TaqMan probes used for these experiments were purchased from Applied Biosystems. Primers were inventoried gene expression assays. TaqMan real-time PCR amplification reactions were performed in a 20 μL reaction mixtures containing 10 μL of TaqMan Universal PCR Master Mix UNG (2X), 9 μL of template cDNA (100 ng/μL), and 1 μL of the corresponding primer/probe mix, using an AB 7500 fast real-time system (Applied Biosystems). For the amplification, the following cycling conditions were applied: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C/1 min at 60°C.

3. Statistical Analysis

All experiments were repeated at least three times. Data were expressed as the mean ± SD or the mean ± SEM. Differences between control and treatments were assessed by Student's unpaired t-test. P values below 0.05, 0.005, and 0.001 were considered significant.

4. Results

4.1. NRE Reduces Cell Differentiation and Lipid Droplet Formation in 3T3-L1 Cells. Adipogenesis assay was performed to investigate the effect of NRE on the adipocyte differentiation and on the lipid droplets accumulation in 3T3-L1 cells using oil Red-O staining. Differentiated 3T3-L1 cells were treated every two days with NRE at various concentration and with 25 μM isorhamnetin (as a positive control), for 7 days. Based on oil Red-O content quantification, results showed that NRE treatment at 25, 50, 100, 200, and 400 μg/mL in 3T3-L1 cells could inhibit the lipid droplet accumulation compared to untreated cells, in dose dependent manner (Figure 1(c)). The triglyceride accumulation significantly decreased to 76.60 ± 7.30%, 69.08 ± 8.08%, 62.90 ± 4.80%, 46.80 ± 7.50%, and 42.70 ± 2.10%, respectively (Figure 1(c)), without any cytotoxic effect (data not shown). At 200 and 400 μg/mL, NRE treatment showed similar effect as isorhamnetin. Moreover, we noticed that NRE treatment in 3T3-L1 was accompanied by modulation of cell hypertrophy rather than cell hyperplasia as indicated in microscopic observation (Figures 1(a) and 1(b)). Thus NRE treatment might induce the cell differentiation into smaller adipocytes compared to untreated cells.

4.2. Antiobesity Effects of NRE in HFD-Induced C57B6J/L Obese Mice. As shown in Figure 2, the HFD increased body weight gain significantly compared to ND over 4 weeks treatment period in C57B6J/L mice. Moreover, final body weight was significantly lower in the HFD + NR50 (24.60 ± 0.50 g) and HFD + NR100 (24.40 ± 0.70 g) groups compared to CHFD group (27.62 ± 0.50 g) (Table 2) ($P < 0.05$), without affecting food intake, which was around 3 g/day/mice for all different experimental groups (Table 3). It is well known that body weight and fat stores are determined by the net excess or deficit of food intake over energy expenditure. In the current study, NRE treatment was demonstrated not only to decrease cumulative body weight gain but also adipose tissue weight and improve adiposity index. In fact 50 mg/kg and 100 mg/kg NRE treatment in HFD mice decreased adipose tissue weight from 1.80 ± 0.20 g in vehicle group to 1.10 ± 0.21 g and 1.20 ± 0.16 g, respectively (Figure 3).

4.3. Effect of NRE Administration on Glucose, Triglycerides, Cholesterol, and TNF-α in HFD-Induced C57B6/JL Obese Mice. Serum glucose and lipid levels (triglycerides, total

Table 2: Body weight gain in ND, HFD, HFD + PC, HFD + NR50, and HFD + NR100 groups for 4 weeks.

	CND	CHFD	HFD + PC	HFD + NR50	HFD + NR100
			Body weight (g)		
Initial	19.12 ± 0.40	19.18 ± 0.30	18.93 ± 0.60	19.40 ± 0.40	19.60 ± 0.40
Final	23.25 ± 0.40	27.62 ± 0.50	26.64 ± 0.20	24.60 ± 0.50*	24.40 ± 0.70*

Data represent the mean ± SEM, $^{*}P < 0.05$, when compared to the CHFD group ($n = 8$ per group).
CND: control normal diet fed group.
CHFD: control high-fat diet fed group.
HFD + PC: high-fat diet + positive control (Naringenin 10 mg/kg body weight).
HFD + NR50: high-fat diet + *Nitraria retusa* 50 mg/kg body weight.
HFD + NR100: high-fat diet + *Nitraria retusa* 100 mg/kg body weight.

Table 3: Food intake (g/day) and food efficiency ratio (FER) in CND, CHFD, HFD + PC, HFD + NR50, and HFD + NR100 groups for 4 weeks.

	CND	CHFD	HFD + PC	HFD + NR50	HFD + NR100
Food intake (g)	3.25 ± 0.10	3.12 ± 0.10	3.00 ± 0.10	3.25 ± 0.20	3.06 ± 0.10
Food efficiency ratio (FER)	1.26 ± 0.07	2.70 ± 0.10	2.42 ± 0.20	1.60 ± 0.07***	1.56 ± 0.10***

Data represent the mean ± SEM, $^{***}P < 0.001$, when compared to the CHFD group ($n = 8$ per group).
CND: control normal diet fed group.
CHFD: control high-fat diet fed group.
HFD + PC: high-fat diet + positive control (Naringenin 10 mg/kg body weight).
HFD + NR50: high-fat diet + *Nitraria retusa* 50 mg/kg body weight.
HFD + NR100: high-fat diet + *Nitraria retusa* 100 mg/kg body weight.

cholesterol, HDL-cholesterol (HDL-c), and LDL-cholesterol (LDL-c)) and also the cytokine TNF-α level of all mice groups were analyzed. Results are summarized in Table 4. The HFD-fed mice showed significant high levels of serum glucose (200.00 ± 4.10 mg/dL), TG (22.00 ± 1.63 mg/dL), and lower level of HDL-c (80.00 ± 1.55 mg/dL) when compared to those fed with normal diet (CND group). As shown in Table 4, 50 mg/kg and 100 mg/kg NRE and Naringenin (positive control) treatments significantly decreased glucose levels in blood serum of HFD-fed mice to reach 168.00 ± 2.69 mg/dL, 153.00 ± 9.41 mg/dL, and 146.00 ± 1.63 mg/dL, respectively. Triglycerides levels were also significantly decreased to 10.00 ± 1.63 mg/dL and 14.00 ± 1.63 mg/dL in 50 mg/kg and 100 mg/kg NRE treated HFD mice, respectively. However, Naringenin treatment did not affect their levels. NRE administration for 4 weeks did not affect the total cholesterol in HFD-induced obese mice blood serum. However, it significantly increased HDL-c fraction (the good cholesterol) from 80.00±1.55 mg/dL in vehicle group to 98.00±4.10 mg/dL, and 92.00 ± 4.10 mg/dL, respectively, for HFD + NR50 and HFD + 100 groups ($P < 0.05$). High-density lipoprotein (HDL) particles transport cholesterol back to the liver for excretion, but vary considerably in their effectiveness for doing this. Having large numbers of large HDL particles correlates with better health outcomes, and hence it is commonly called "good cholesterol." On the other hand, only 100 mg/kg NRE administration, significantly, decreased LDL-c (the bad cholesterol) from 16.50 ± 2.03 mg/dL for vehicle group to 12.00 mg/dL ($P < 0.05$).

4.4. Effect of NRE Treatment on Hepatic Lipid Metabolism Gene Expression in HFD-Induced C57B6/JL Obese Mice. High serum lipid level mainly triglycerides and cholesterol are a hallmark of many metabolic syndrome diseases such as type 2 diabetes. Understanding the molecular mechanism that undergoes dyslipidemia should facilitate improved strategies for serum lipid management. In this respect, the effect of NRE administration in HFD-induced mice on hepatic lipid metabolism biomarkers at translational level was investigated, following 4 weeks experimental study.

In fact, 50 mg/kg and 100 mg/kg NRE treatments significantly increased the gene expression of the hepatic PPAR γ 1 (Figure 4(a)) to 6 and 4 folds, respectively. While only 50 mg/kg NRE dose improved PPAR α gene expression (Figure 4(b)) by 2 folds. Moreover, NRE treatments significantly modulated the lipogenic enzyme genes ACC1 and FAS. In fact FAS gene expression decreased by half at 100 mg/kg dose (Figure 5(a)), and ACC1 gene expression increased by 2.5 folds at 50 mg/kg dose (Figure 5(b)). Additionally, 50 mg/kg NRE treatment in HFD-induced obese C57BL/6J mice significantly increased CPT1 (Figure 6) and LPL (Figure 7) gene expression to reach 6 and 30 folds, respectively. These results were concordant with weight loss, adiposity index, and biochemical metabolites investigation. The current data indicates that *in vivo* administration of NRE could be an effective plant preparation in enhancing liver lipid metabolism and preventing obesity.

5. Discussion

In the current study, NRE treatment in differentiated adipocyte 3T3-L1 cells significantly inhibited lipid droplet accumulation and modulated cell hypertrophy and not cell hyperplasia in a similar trend as isorhamnetin treatment. In fact, the adipocyte is the primary site of energy storage and triglycerides accumulation during nutritional excess.

Table 4: Blood constituents in CND, CHFD, HFD + PC, HFD + NR50, and HFD + NR100 groups after 4 weeks.

	CND	CHFD	HFD + PC	HFD + NR50	HFD + NR100
Serum total cholesterol (mg/dL)	134.00 ± 4.65	174.00 ± 8.64	154.00 ± 4.10	182.00 ± 1.55	170.00 ± 5.59
Serum HDL-cholesterol (mg/dL)	66.00 ± 4.65	80.00 ± 1.55	64.00 ± 1.55**	98.00 ± 4.10*	92.00 ± 4.10
Serum LDL-cholesterol (mg/dL)	9.00 ± 1.22	16.50 ± 2.03	6.00 ± 0.00**	18.00 ± 0.00	12.00 ± 0.00*
Serum triglycerides (mg/dL)	16.00 ± 3.11	22.00 ± 1.63	22.00 ± 1.63	10.00 ± 1.63*	14.00 ± 1.63*
Serum glucose (mg/dL)	102.00 ± 14.97	200.00 ± 4.10	146.00 ± 1.63**	168.00 ± 2.69**	153.00 ± 9.41*
Serum TNF-α (pg/mL)	63.20 ± 1.40	61.08 ± 0.58	61.31 ± 0.58	64.43 ± 0.56	65.72 ± 1.22

Data represent the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.005$, when compared to the CHFD group ($n = 5$ per group).
CND: control normal diet fed group.
CHFD: control high-fat diet fed group.
HFD + PC: high-fat diet + positive control (Naringenin 10 mg/kg body weight).
HFD + NR50: high-fat diet + *Nitraria retusa* 50 mg/kg body weight.
HFD + NR100: high-fat diet + *Nitraria retusa* 100 mg/kg body weight.

Moreover, NRE administrations in HFD-induced obese C57BL/6J mice for 4 consecutive weeks significantly reduced body weight gain and adipose tissue accumulation without affecting food intake. Furthermore, these effects were in concordance with a significant improvement of glucose and lipid metabolism in blood serum and the expression profiles of genes related to beta-oxidation, lipolysis, and lipogenesis in the liver. These findings demonstrated that NRE suppresses obesity in HFD-induced obese mice.

It is well known that obesity is caused by imbalanced homeostasis between low energy expenditure and increased energy intake and accumulation [22]. Excess energy is mainly stored as triglycerides in adipose tissue which increase the visceral adipose tissue mass through adipocyte hypertrophy and hyperplasia. Several strategies are proposed to reduce or suppress obesity, among them dietary supplements and natural products. In fact, herbal and botanical preparations are gaining a lot of interest either to substitute chemical drugs or to be combined with them. In this respect, *Nitraria retusa*, an edible halophyte plant growing wild in Tunisia, could be a potent candidate. Previous phytochemical studies from our research and others demonstrated that *Nitraria retusa* has a strong anti-oxidant and free radical scavenging properties due mainly to its high contents in polyphenols and flavonoids [23]. The HPLC analysis showed the presence of several alkaloids like 5, 7-dihydroxy-3-deoxy-vasiciene I, 7-hydroxy-3-deoxyl-vasiciene II, and O-acetylnitraraine I [18]. The phenolic profile showed mainly, high contents of isorhamnetin aglycone and glycosides [23] and other flavonoids like apigenin, quercetin, kaempferol, and luteolin [24]. The high content in flavonoids and their possible synergetic effect may explain in part the antiobesity effect of NRE, since these compounds were individually demonstrated to have high potential to prevent metabolic syndrome diseases [25] and their mixture showed synergetic antiobesity effect [26–33].

The investigation of biochemical markers like glucose, total triglycerides, total cholesterol, LDL, HDL, and TNF-α in mice blood serum demonstrated that NRE significantly improved these parameters except for TNF-α and overall data showed higher activity than Naringenin, commonly used as positive control. In general, the accumulation of triglycerides in the liver is due to an imbalance between the availability of hepatic triglycerides for export and the exporting capacity of the liver via VLDLs [34]. Furthermore, in the liver cells an increase in glucose exerts, both directly and indirectly, a series of effects which result in the orientation of its metabolism towards glycogen synthesis, glycolysis, and formation of fatty acids [6]. On the one hand a first direct effect of glucose is to stimulate its hepatic uptake which could be ameliorated with NRE activity on the lowering effect of the serum glucose level. On the other hand, an increase in triglycerides levels, particularly when accompanied by a decrease in high-density lipoprotein (HDL) levels, has been shown to be a surrogate marker of insulin resistance, a strong predisposing condition for type 2 diabetes [35].

Lipids and carbohydrates metabolism in liver is controlled by several genes. In this respect, the investigations of NRE treatment in HFD-induced obese mice on hepatic genes related to beta-oxidation, lipolysis, and lipogenesis showed a significant improvement of their expression when compared to vehicle group or Naringenin treatment. In fact, Naringenin was reported to ameliorate hepatic steatosis and attenuate dyslipidemia, without affecting caloric absorption [36] with an improvement of hepatic fatty acid oxidation through PPAR α coactivator 1 alpha.

Fatty acid metabolism in the liver involves three main pathways: catabolism by β-oxidation, synthesis, from acetyl CoA, and esterification into triglycerides. Herein in our study, NRE administration in HFD-fed mice significantly overexpressed PPAR α by 2 folds increase and promoted fatty acid β-oxidation, CPT1 gene, by 6 folds. PPAR family has been demonstrated to be highly expressed in the parenchymal cells of the liver in relation to lipid catabolism and storage. In fact, PPAR α is homogenous group of genes that participate in lipid catabolism such as fatty acid uptake through membrane, fatty acid binding in cells, fatty acid oxidation, and lipoprotein assembly and transport. PPAR γ 1 is known to influence the storage of fatty acids in the adipose tissue [37], but its mRNA expression is detected at lower level in liver. This in turn could be one of other factors (period of high-fat diet feeding…) affecting its expression in liver of mice fed

with HFD compared to those fed with ND (Figure 4(a)). PPAR α activation is known to mediate the expression of genes promoting fatty acid β-oxidation mainly CPT1 gene. CPT1 is the encoding gene of carnitine palmitoyltransferase system which is a critical and essential step in the β-oxidation of long chain fatty acids. Such cascade of molecular events will lead finally to lowering the circulating fatty acids and triglycerides-rich lipoproteins [38]. Furthermore, NRE administration in HFD-fed mice significantly increased ACC1 gene expression encoding for the lipogenic enzyme Acetyl-CoA Carboxylase and slightly decreased FAS gene expression encoding for the fatty acid synthase at 50 mg/kg dose. Such effect demonstrates that NRE administration did not negatively affect the fatty acid metabolism since there was an enhancement of CPT1 expression in the liver of NRE-treated mice. ACC has critical roles in fatty acid metabolism and represents an attractive target for therapeutic uses in the control of obesity [39].

Regarding the gene expression of LPL that encodes the enzyme responsible for the hydrolysis of triglycerides in lipoproteins and its effect on the plasma cholesterol level, results showed that the oral administration of NRE in mice fed with high-fat diet had a highly significant overexpression of LPL more than 30-fold. In this regard, it has been demonstrated that LPL overexpression prevents the development of diet-induced hypertriglyceridemia and hypercholesterolemia and decreases VLDL and LDL fractions levels [40]. On the other hand, previous study revealed that plasma cholesterol levels were decreased in LPL transgenic mice after cholesterol loading. These findings suggest that LPL plays an important role in determining cholesterol levels. Furthermore, it has been also highlighted that free fatty acids uptake into adipocytes is also facilitated by the extracellular expression and activity of lipoprotein lipase [41]. LPL activity changes dramatically in various tissues in response to energy requirements and its systemic overexpression results in increases in whole body insulin sensitivity.

Our study demonstrated that NRE treatment in HFD fed mice significantly ameliorated the hepatic gene profile expression involved in energy homeostasis (glucose and lipid metabolism). In this respect several herbal preparations cited in the literature showed similar effects. Flavonoids like isorhamnetin [42], Naringenin [43], and quercetin [44] individually or mixed showed significant reduction in obesity and type 2 diabetes incidence.

Taken together, our results demonstrated that NRE treatment at *in vitro* and *in vivo* levels exerts antiobesity action through lowering glucose and triglycerides and the enhancement of the lipid metabolism in liver due to the increasing of serum HDL-cholesterol and the decreasing of LDL-cholesterol modulating the gene expression related to lipid metabolism. This effect may be due to the improvement of the antioxidant status within hepatic cells by the strong antioxidant activities of many phenolic components present in NRE especially flavonoids such as isorhamnetin aglycones and glycosides. Thus, the identification of possible active compounds and standardization of NRE may provide an opportunity to develop a novel class of antiobesity supplement or functional food. Further investigations will be needed in order to evaluate NRE antiobesity bioactive molecules efficacy and their bioavailability.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Traditional Medicines in Africa: An Appraisal of Ten Potent African Medicinal Plants

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The use of medicinal plants as a fundamental component of the African traditional healthcare system is perhaps the oldest and the most assorted of all therapeutic systems. In many parts of rural Africa, traditional healers prescribing medicinal plants are the most easily accessible and affordable health resource available to the local community and at times the only therapy that subsists. Nonetheless, there is still a paucity of updated comprehensive compilation of promising medicinal plants from the African continent. The major focus of the present review is to provide an updated overview of 10 promising medicinal plants from the African biodiversity which have short- as well as long-term potential to be developed as future phytopharmaceuticals to treat and/or manage panoply of infectious and chronic conditions. In this endeavour, key scientific databases have been probed to investigate trends in the rapidly increasing number of scientific publications on African traditional medicinal plants. Within the framework of enhancing the significance of traditional African medicinal plants, aspects such as traditional use, phytochemical profile, *in vitro*, *in vivo*, and clinical studies and also future challenges pertaining to the use of these plants have been explored.

1. Introduction

Traditional medicine is the sum total of knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve, or treat physical and mental illnesses [1]. Traditional medicine that has been adopted by other populations (outside its indigenous culture) is often termed complementary or alternative medicine (CAM) [1, 2].

The World Health Organization (WHO) reported that 80% of the emerging world's population relies on traditional medicine for therapy. During the past decades, the developed world has also witnessed an ascending trend in the utilization of CAM, particularly herbal remedies [3]. Herbal medicines include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant materials as active ingredients. While 90% of the population in Ethiopia use herbal remedies for their primary healthcare, surveys carried out in developed countries like Germany and Canada tend to show that at least 70% of their population have tried CAM at least once [2, 3]. It is likely that the profound knowledge of herbal remedies in traditional cultures, developed through trial and error over many centuries, along with the most important cures was carefully passed on verbally from one generation to another. Indeed, modern allopathic medicine has its roots in this ancient medicine, and it is likely that many important new remedies will be developed and commercialized in the future from the African biodiversity, as it has been till now, by following the leads provided by traditional knowledge and experiences [2–5].

The extensive use of traditional medicine in Africa, composed mainly of medicinal plants, has been argued to be linked to cultural and economic reasons. This is why the WHO encourages African member states to promote and integrate traditional medical practices in their health system [1]. Plants typically contain mixtures of different phytochemicals, also known as secondary metabolites that may act individually, additively, or in synergy to improve health. Indeed, medicinal plants, unlike pharmacological drugs, commonly have several chemicals working together catalytically and synergistically to produce a combined effect that surpasses the total activity of the individual constituents.

The combined actions of these substances tend to increase the activity of the main medicinal constituent by speeding up or slowing down its assimilation in the body. Secondary metabolites from plant's origins might increase the stability of the active compound(s) or phytochemicals, minimize the rate of undesired adverse side effects, and have an additive, potentiating, or antagonistic effect. It has been postulated that the enormous diversity of chemical structures found in these plants is not waste products, but specialized secondary metabolites involved in the relationship of the organism with the environment, for example, attractants of pollinators, signal products, defensive substances against predators and parasites, or in resistance against pests and diseases. A single plant may, for example, contain bitter substances that stimulate digestion and possess anti-inflammatory compounds that reduce swellings and pain, phenolic compounds that can act as an antioxidant and venotonics, antibacterial and antifungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste products and toxins, and alkaloids that enhance mood and give a sense of well-being [1–5]. Although some may view the isolation of phytochemicals and their use as single chemical entities as a better alternative and which have resulted in the replacement of plant extracts' use, nowadays, a view that there may be some advantages of the medical use of crude and/or standardized extracts as opposed to isolated single compound is gaining much momentum in the scientific community.

2. African Traditional Medicine

African traditional medicine is the oldest, and perhaps the most assorted, of all therapeutic systems. Africa is considered to be the cradle of mankind with a rich biological and cultural diversity marked by regional differences in healing practices [2, 6]. African traditional medicine in its varied forms is holistic involving both the body and the mind. The traditional healer typically diagnoses and treats the psychological basis of an illness before prescribing medicines, particularly medicinal plants to treat the symptoms [2, 6–8]. The sustained interest in traditional medicine in the African healthcare system can be justified by two major reasons. The first one is inadequate access to allopathic medicines and western forms of treatments, whereby the majority of people in Africa cannot afford access to modern medical care either because it is too costly or because there are no medical service providers. Second, there is a lack of effective modern medical treatment for some ailments such as malaria and/or HIV/AIDS, which, although global in distribution, disproportionately affect Africa more than other areas in the world.

The most common traditional medicine in common practice across the African continent is the use of medicinal plants. In many parts of Africa, medicinal plants are the most easily accessible health resource available to the community. In addition, they are most often the preferred option for the patients. For most of these people, traditional healers offer information, counseling, and treatment to patients and their families in a personal manner as well as having an understanding of their patient's environment [2, 6, 7]. Indeed, Africa is blessed with enormous biodiversity resources and it is estimated to contain between 40 and 45,000 species of plant with a potential for development and out of which 5,000 species are used medicinally. This is not surprising since Africa is located within the tropical and subtropical climate and it is a known fact that plants accumulate important secondary metabolites through evolution as a natural means of surviving in a hostile environment [9]. Because of her tropical conditions, Africa has an unfair share of strong ultraviolet rays of the tropical sunlight and numerous pathogenic microbes, including several species of bacteria, fungi, and viruses, suggesting that African plants could accumulate chemopreventive substances more than plants from the northern hemisphere. Interestingly, Abegaz et al. [10] have observed that of all species of *Dorstenia* (Moraceae) analysed, only the African species, *Dorstenia mannii* Hook.f, a perennial herb growing in the tropical rain forest of Central Africa contained more biological activity than related species [9–11].

Nonetheless, the documentation of medicinal uses of African plants and traditional systems is becoming a pressing need because of the rapid loss of the natural habitats of some of these plants due to anthropogenic activities and also due to an erosion of valuable traditional knowledge. It has been reported that Africa has some 216 million hectares of forest, but the African continent is also notorious to have one of the highest rates of deforestation in the world, with a calculated loss through deforestation of 1% per annum [7, 12]. Interestingly, the continent also has the highest rate of endemism, with the Republic of Madagascar topping the list by 82%, and it is worth to emphasize that Africa already contributes nearly 25% of the world trade in biodiversity. Nonetheless, the paradox is that in spite of this huge potential and diversity, the African continent has only few drugs commercialized globally [2, 12, 13].

The scientific literature has witnessed a growing number of publications geared towards evaluating the efficacy of medicinal plants from Africa which are believed to have an important contribution in the maintenance of health and in the introduction of new treatments. Nonetheless, there is still a dearth of updated comprehensive compilation of promising medicinal plants from the African continent.

The main aim of the present review is to highlight the importance and potential of medicinal plants from the African biodiversity which have short- as well as long-term potential to be developed as future phytopharmaceuticals to treat and/or manage panoply of infectious and chronic conditions. The review might also provide a starting point for future studies aimed at isolation, purification, and characterization of bioactive compounds present in these plants as well as exploring the potential niche market of these plants. In this endeavor, major scientific databases such as EBSCOhost, PubMed Central, Scopus (Elsevier), and Emerald amongst others have been probed to investigate trends in the rapidly increasing number of scientific publications on African traditional medicinal plants. Ten medicinal plants (*Acacia senegal*, *Aloe ferox*, *Artemisia herba-alba*, *Aspalathus linearis*, *Centella asiatica*, *Catharanthus roseus*, *Cyclopia genistoides*, *Harpagophytum procumbens*, *Momordica charantia*, and *Pelargonium*

sidoides) of special interest were chosen for more detailed reviews based on the following criteria: medicinal plants that form part of African herbal pharmacopeia with commercial importance and those plants from which modern phytopharmaceuticals have been derived.

2.1. Acacia senegal (L.) Willd. (Leguminosae: Mimosoideae)—Gum Arabic. *Acacia senegal*, also known as gum Arabic, is native to semidesert and drier regions of sub-Saharan Africa, but widespread from Southern to Northern Africa. It is used as a medicinal plant in parts of Northern Nigeria, West Africa, North Africa, and other parts of the world [8]. The use of gum arabic (or gum acacia), which is derived from an exudate from the bark, dates from the first Egyptian Dynasty (3400 B.C.). It was used in the production of ink, which was made from a mixture of carbon, gum, and water. Inscriptions from the 18th Dynasty refer to this gum as "komi" or "komme." Gum arabic has been used for at least 4,000 years by local people for the preparation of food, in human and veterinary medicine, in crafts, and as a cosmetic. The gum of *A. senegal* has been used medicinally for centuries, and various parts of the plant are used to treat infections such as bleeding, bronchitis, diarrhea, gonorrhea, leprosy, typhoid fever, and upper respiratory tract infections. African herbalists use gum acacia to bind pills and to stabilize emulsions. It is also used in aromatherapy for applying essential oils [8, 14–16].

Currently, *A. senegal* is an important naturally occurring oil-in-water emulsifier, which is in regular use in the food and pharmaceutical industries. Medicinally, gum arabic is used extensively in pharmaceutical preparations and is a food additive approved as toxicologically safe by the Codex Alimentarius. It has been used as demulcent, skin protective agent, and pharmaceutical aids such as emulsifier and stabilizer of suspensions and additives for solid formulations. It is sometimes used to treat bacterial and fungal infections of the skin and mouth. It has been reported to soothe the mucous membranes of the intestines and to treat inflamed skin [17, 18]. The demulcent, emollient gum is used internally against inflammation of intestinal mucosa and externally to cover inflamed surfaces, as burns, sore nipples, and nodular leprosy. Additionally, it has also been documented to be used as antitussive, expectorant, astringent, catarrh and against colds, coughs, diarrhea, dysentery, gonorrhea, hemorrhage, sore throat, typhoid, and for urinary tract ailments [18]. The gum of *A. senegal* has been pharmaceutically used mainly in the manufacture of emulsions and in making pills and troches (as an excipient), as demulcent for inflammations of the throat or stomach and as masking agent for acrid tasting substances such as capsicum and also as a film-forming agent in peel-off masks. Gum arabic is also used widely as an ingredient in foods like candies and soft drinks as the gum has the properties of glue that is safe to eat. Gum acacia is widely used in organic products as natural alternative to chemical binders and is used in commercial emulsification for the production of beverages and flavor concentrates [8, 17–19].

Recently, it has been reported that *A. senegal* bark extracts were evaluated *in vitro* for their antimicrobial potential against human pathogenic isolates (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Salmonella typhi*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*). The extract was found to exhibit significant antibacterial activity which was suggested to be due to the presence of tannins and saponins in the plant. It was also reported that the plant extract may not be toxic to man following *in vitro* cytotoxicity evaluation [18].

2.2. Aloe ferox Mill. (Xanthorrhoeaceae)—Bitter Aloe or Cape Aloe. *Aloe ferox* is native to South Africa and Lesotho and is considered to be the most common Aloe species in South Africa. *A. ferox* has been used since time immemorial and has a well-documented history of use as an alternative medicine and is one of the few plants depicted in San rock paintings. The bitter latex, known as Cape aloe, is used as laxative medicine in Africa and Europe and is considered to have bitter tonic, antioxidant, anti-inflammatory, antimicrobial, and anticancer properties [8, 19–23].

The use of *A. ferox* as a multipurpose traditional medicine has been translated into several commercial applications and it is a highly valued plant in the pharmaceutical, natural health, food, and cosmetic industries. *A. ferox* is considered South Africa's main wild harvested commercially traded species. The finished product obtained from aloe tapping, aloe bitters, has remained a key South African export product since 1761 when it was first exported to Europe. The aloe tapping industry is the livelihood of many rural communities and formalization of the industry in the form of establishment of cooperatives and trade agreements. It has been suggested that its trade may have an extensive poverty alleviation effect in Africa [19, 24, 25].

A. ferox has many traditional and documented medicinal uses. It is most popularly used for its laxative effect and as a topical application to the skin, eyes, and mucous membranes. Scientific studies conducted have verified many of the traditional uses. More recently, the cosmetic industry has shown interest in *A. ferox* gel [8, 19]. It has been reported that *A. ferox* gel contains at least 130 medicinal agents with anti-inflammatory, analgesic, calming, antiseptic, germicidal, antiviral, antiparasitic, antitumour, and anticancer effects encompassing all of the traditional uses and scientific studies done on *A. ferox* and its constituents [8, 24–31].

A wide variety of phenolic compounds (chromones, anthraquinones, anthrone, anthrone-C-glycosides, and other phenolic compounds) are present within *A. ferox* and these compounds have been well documented to possess biological activity [21, 24–31]. The gel polysaccharides are known to be of the arabinogalactan and rhamnogalacturonan types. The leaf gel composition still remains unknown and its claimed biological activities still remain to be investigated. The active ingredient (purgative principle) is a chemical compound known as Aloin (also called Barbaloin) [19]. The gel has also been found to be rich in antioxidant polyphenols, indoles, and alkaloids. Tests carried out have shown that the nonflavonoid polyphenols contribute to the majority of the total polyphenol content. With this phytochemical profile, *A. ferox* leaf gel has been identified to be very promising in alleviating symptoms associated with/or prevention of

common noncommunicable diseases such as cardiovascular diseases, cancer, neurodegeneration, and diabetes [32].

The leaves have been reported to contain two juices; the yellow bitter sap is used as laxative while the white aloe gel is used in health drinks and skin care products. This purgative drug is used for stomach complaints, mainly as a laxative to "purify" the stomach and also as a bitter tonic (*amarum*) in various digestives and stomachics (such as "Lewensessens" and "Swedish Bitters"). Usually, a small crystal of the drug (0.05–0.2 g) is taken orally as a laxative. Half the laxative dose is suggested for arthritis. The fresh bitter sap is instilled directly against conjunctivitis and sinusitis [24, 25]. It is well known that bitter substances stimulate the flow of gastric juices and in so doing improve digestion. The fresh juice emanating from the cut leaf is also applied on burn wounds [33]. *A. ferox* is claimed to detoxify the damaged surface area and exhibit analgesic and anesthetic properties while promoting new tissue formation (granulation) which fills the wound. It was demonstrated that *A. ferox* enriched with aloins can inhibit collagenase and metalloprotease activity, which can degrade collagen connective tissues. The effect of *A. ferox* whole leaf juice on wound healing and skin repair was investigated in an animal model and its safety was evaluated. The results showed that the *A. ferox* whole leaf juice preparation accelerates wound closure and selectively inhibits microbial growth. No dermal toxicity or side effects were observed during the experimental period [23].

2.3. Artemisia herba-alba Asso (Med)—Asteraceae—Wormwood. Artemisia herba-alba is commonly known as wormwood or desert wormwood (known in Arabic as shih, and as Armoise blanche in French). It is a greyish strongly aromatic perennial dwarf shrub native to the Northern Africa, Arabian Peninsula, and Western Asia [34]. *A. herba-alba* has been used in folk medicine by many cultures since ancient times. In Moroccan folk medicine, it is used to treat arterial hypertension and diabetes and in Tunisia, it is used to treat diabetes, bronchitis, diarrhea, hypertension, and neuralgias [34, 35]. Herbal tea from *A. herba-alba* has been used as analgesic, antibacterial, antispasmodic, and hemostatic agents in folk medicines [34–39]. During an ethnopharmacological survey carried out among the Bedouins of the Negev desert, it was found that *A. herba-alba* was used to mitigate stomach disorders. This plant is also suggested to be important as a fodder for sheep and for livestock in the plateau regions of Algeria where it grows abundantly. It has also been reported that Ascaridae from hogs and ground worms were killed by the oil of the Libyan *A. herba-alba* in a short time [34–42].

Oral administration of 0.39 g/kg body weight of the aqueous extract of the leaves or barks of *A. herba-alba* has been documented to produce a significant reduction in blood glucose level, while the aqueous extract of roots and methanolic extract of the aerial parts of the plant produce almost no reduction in blood glucose level. The extract of the aerial parts of the plant seems to have minimal adverse effect and high LD_{50} value [19, 30].

Among *A. herba-alba* phytochemical constituents, essential oils have been extensively studied, with several chemotypes being recognized. The variability from the essential oils isolated from *A. herba-alba* collected in Algeria, Israel, Morocco, and Spain was revised by Dob and Benabdelkader [43, 44], but, since then, many other studies have reinforced its high chemical polymorphism. Recently, fifty components were identified in *A. herba-alba* oils, oxygen-containing monoterpenes being dominant in all cases (72–80%). Camphor (17–33%), α-thujone (7–28%), and chrysanthenone (4–19%) were the major oil components. Despite the similarity in main components, three types of oils could be defined: (a) α-thujone : camphor (23–28 : 17–28%), (b) camphor : chrysanthenone (33 : 12%), and (c) α-thujone : camphor : chrysanthenone (24 : 19 : 19%) [43].

The antifungal activity of *Artemisia herba-alba* was found to be associated with two major volatile compounds isolated from the fresh leaves of the plant. Carvone and piperitone were isolated from *Artemisia herba-alba*. The antifungal activity of the purified compounds carvone and piperitone was estimated to be 5 μg/mL and 2 μg/mL against *Penicillium citrinum* and 7 μg/mL and 1.5 μg/mL against *Mucor rouxii*, respectively. In another study, the antifungal activity of the constituents and biological activities of *Artemisia herba-alba* essential oils of 25 Moroccan medicinal plants, including *A. herba-alba*, against *Penicillium digitatum*, *Phytophthora citrophthora*, *Geotrichum citri-aurantii*, and *Botrytis cinerea* have been reported [42, 43].

2.4. Aspalathus linearis (Brum.f.) R. Dahlg. (Fabaceae)—Rooibos. Aspalathus linearis, an endemic South African fynbos species, is cultivated to produce the well-known herbal tea, also commonly known as rooibos. Its caffeine-free and comparatively low tannin status, combined with its potential health-promoting properties, most notably antioxidant activity, has contributed to its popularity and consumer acceptance globally. The utilization of rooibos has also moved beyond a herbal tea to intermediate value-added products such as extracts for the beverage, food, nutraceuticals and cosmetic markets [45–52].

Rooibos is used traditionally throughout Africa in numerous ways. It has been used as a refreshment drink and as a healthy tea beverage [8, 19]. It was only after the discovery that an infusion of rooibos, when administered to her colicky baby, cured the chronic restlessness, vomiting, and stomach cramps that rooibos became well known as a "healthy" beverage, leading to a broader consumer base. Many babies since then have been nurtured with rooibos—either added to their milk or given as a weak brew [8, 16, 45–52].

Animal studies have suggested that it has potent antioxidant, immunomodulating, and chemopreventive effects. The plant is rich in minerals and low in tannins. Among the flavonoids present are the unique C-glucoside dihydrochalcones: aspalathin and nothofagin and with aspalathin being the most abundant. *In vitro* data has shown that the daily intake of the alkaline extracts of the red rooibos tea could suppress HIV infections in the extract, though clinical evaluation has yet to be conducted [8, 45]. There is growing evidence that the flavonoids present in the plant contribute substantially to a reduction in cardiovascular disease and other ailments associated with ageing. Recent studies have shown that aspalathin has beneficial effects on glucose

homeostasis in Type 2 diabetes through stimulating glucose uptake in muscle tissues and insulin secretion from pancreatic beta-cells [8, 48]. The unfermented rooibos has been found to have greater chemoprotective effects than the fermented variety [49]. Aspalathin has free-radical capturing properties and is absorbed through the small intestine as such [50].

The bronchodilator, antispasmodic, and blood pressure lowering effects of rooibos tea have been confirmed *in vitro* and *in vivo*. It has also been reported that the antispasmodic effect of the rooibos is mediated predominantly through potassium ionchannel activation [51, 52]. There is also increasing evidence of antimutagenic effects. Animal study suggested the prevention of age-related accumulation of lipid peroxidases in the brain [19, 26, 47].

Rooibos is becoming more popular in western countries particularly among health-conscious consumers, due to the absence of alkaloids and low tannin content. It is also reported to have a high level of antioxidants such as aspalathin and nothofagin. The antioxidative effect has also been attributed to the presence of water-soluble polyphenols such as rutin and quercetin [53]. Rooibos is purported to assist nervous tension, allergies, and digestive problems.

Rooibos extracts, usually combined with other ingredients, are available in pill form, but these products fall in the category of dietary supplements. Recent research has underscored the potential of aspalathin and selected rooibos extracts such as an aspalathin-enriched green rooibos extract as antidiabetic agents [54–68]. A patent application for the use of aspalathin in this context was filed in Japan, while a placebo-controlled trial application was filed for the use of rooibos extract as an antidiabetic agent [66]. It is claimed that rooibos extract and a hetero-dimer containing aspalathin, isolated from rooibos, could be used as a drug for the treatment of neurological and psychiatric disorders of the central nervous system [19, 68]. Other opportunities may lie with topical skin products. Two studies concerning inhibition of COX-2 in mouse skin [67] and inhibition of mouse skin tumor promotion tend to support the role of the topical application of rooibos extract. Nonetheless, more research would be needed to explore its potential in preventing skin cancer in humans [67].

2.5. Centella asiatica (L.) Urb. (Apiaceae)—Centella. *Centella asiatica* is a medicinal plant that has been used since prehistoric times. It has a pan-tropical distribution and is used in many healing cultures, including Ayurvedic medicine, Chinese traditional medicine, Kampo (Japanese traditional medicine), and African traditional medicine [19, 69]. To date, it continues to be used within the structure of folk medicine and is increasingly being located at the interface between traditional and modern scientifically oriented medicine. Traditionally, *C. asiatica* is used mainly for wound healing, burns, ulcers, leprosy, tuberculosis, lupus, skin diseases, eye diseases, fever, inflammation, asthma, hypertension, rheumatism, syphilis, epilepsy, diarrhea, and mental illness and is also eaten as a vegetable or used as a spice. In Mauritius, the application of *C. asiatica* in the treatment of leprosy was reported for the first time in 1852 while the clinical use of *C. asiatica*, as a therapeutic agent suitable for the treatment of leprous lesions, has been documented since 1887 [19].

The active constituents are characterized by their clinical effects in the treatment of chronic venous disease, wound healing, and cognitive functions amongst others [19]. *C. asiatica* contains a variety of pentacyclic triterpenoids that have been extensively studied. Asiaticoside and madecassoside are the two most important active compounds that are used in drug preparations. Both are commercially used mainly as wound-healing agent, based on their anti-inflammatory effects. One of the main active constituents of *C. asiatica* is the ursane-type triterpene saponin, asiaticoside, which is responsible for wound healing properties [19, 70, 71] and is known to stimulate type 1 collagen synthesis in fibroblast cells [72]. Plants collected from various geographical regions and locations in India, Madagascar, Malaysia, Sri Lanka, Andaman Islands, and South Africa have yielded concentrations of asiaticoside ranging from 0.006 to 6.42% of dry weight [73, 74]. *C. asiatica* also contains several other triterpene saponins. Madecassoside always co-occurs with asiaticoside as a main compound and other saponins have been reported, such as asiaticosides A to G, centelloside, brahmoside, and many others [19, 75]. Madagascar plays a major role in *C. asiatica* trade. It is the first producer of *C. asiatica* products worldwide and due to a higher Asiaticoside content of dried leaves, Malagasy origin is appreciated by industry [9].

The ethyl acetate fraction of *C. asiatica* has been reported to increase the effect of the *i.p.* administrated antiepileptic drugs phenytoin, valproate, and gabapentin [75, 76] and was found to decrease the pentylenetetrazol- (PTZ-) kindled induced seizures in rats [75, 77]. This effect might be due to an increase in gamma-aminobutyric acid (GABA) levels caused by the extract as reported by Chatterjee et al. [78]. The neuroprotective properties of the plant in monosodium glutamate treated rats were investigated by Ramanathan et al. [79]. The general behavior, locomotor activity, and the CA1 region of the hippocampus were protected by *C. asiatica* extracts. The levels of catalase, superoxide dismutase, and lipid peroxidase in the hippocampus and striatum were improved indicating a neuroprotective property of the extract [74]. Additionally, the effect of *C. asiatica* on cognitive function of healthy elderly volunteer was evaluated in a randomized, placebo-controlled, double-blind study involving 28 healthy elderly participants. The subjects have received the plant extract at various doses ranging from 250 to 500 and 750 mg once daily for 2 months, and cognitive performance and mood modulation were assessed. It was found that high dose of the plant extract enhanced working memory and increased N100 component amplitude of event-related potential. Improvements of self-rated mood were also found following the *C. asiatica* treatment. The high dose of *C. asiatica* used in the study was suggested to increase calmness and alertness after 1 and 2 months of treatment. However, the precise mechanism(s) underlying these effects still require further investigation [72].

2.6. Catharanthus roseus (L.) G. Don (Apocynaceae)—Madagascan Periwinkle. *Catharanthus roseus* (Madagascar

periwinkle) is a well-known medicinal plant that has its root from the African continent. The interest in this species arises from its therapeutic role, as it is the source of the anticancer alkaloids vincristine and vinblastine, whose complexity renders them impossible to be synthesized in the laboratory; the leaves of this species are still, today, the only source [8, 12, 19, 80]. *C. roseus* originates from Madagascar but now has a wide distribution throughout the tropics, and the story on the traditional utilisation of this plant can be retraced to Madagascar where healers have been using it extensively to treat panoply of ailments. It is commonly used in traditional medicine as a bitter tonic, galactogogue, and emetic. Application for treatment of rheumatism, skin disorders, and venereal diseases has also been reported [8, 10, 19].

C. roseus has been found to contain a plethora of phytochemicals (as many as 130 constituents) and the principal component is vindoline (up to 0.5%). Other biologically active compounds are serpentine, catharanthine, ajmalicine (raubasine), akuammine, lochnerine, lochnericine, and tetrahydroalstonine. The plant is also rich in bisindole alkaloids; vindoline and catharanthine are found in very small amounts: vincristine (=leurocristine) in up to 3 g/t of dried drug and vinblastine (=vincaleucoblastine) in a slightly larger amount [8, 12, 19, 81–84].

The oral administration of water-soluble fractions and ethanolic extracts of the leaves have been found to show significant dose-dependent reduction in the blood sugar at 4 h by 26.2, 31.4, 35.6, and 33.4%, respectively, in normal rats. In addition, oral administration of 500 mg/kg 3.5 h before oral glucose tolerance test (10 mg/kg) and 72 h after STZ administration (50 mg/kg IP) in rats showed significant antihyperglycaemic effects. No gross behavioural changes and toxic effects were observed up to 4 mg/kg IP [85].

Extract at dose of 500 mg/kg given orally for 7 and 15 days showed 48.6 and 57.6% hypoglycemic activity, respectively. Prior treatment at the same dose for 30 days provided complete protection against streptozotocin (STZ) challenge (75 mg/kg/i.p. × 1). Enzymatic activities of glycogen synthase, glucose 6-phosphate-dehydrogenase, succinate dehydrogenase, and malate dehydrogenase were decreased in liver of diabetic animals in comparison to normal ones and were significantly improved after treatment with extract at dose of 500 mg/kg p.o. for 7 days. Results indicate increased metabolization of glucose in treated rats. Increased levels of lipid peroxidation measured as 2-thiobarbituric acid reactive substances indicative of oxidative stress in diabetic rats were also normalized by treatment with the extract [2].

Vincristine and Vinblastine are antimitotics as they bind to tubulin and prevent the formation of microtubules that assist in the formation of the mitotic spindle; in this way, they block mitosis in the metaphase. These alkaloids are highly toxic; they both have neurotoxic activity (especially vincristine) because the microtubule assembly also plays a role in neurotransmission. Their peripheral neurotoxic effects are neuralgia, myalgia, paresthesia, loss of the tendon reflexes, depression, and headache, and their central neurotoxic effects are convulsive episodes and respiratory difficulties. Other side effects are multiple and include alopecia, gastrointestinal distress including constipation, ulcerations of the mouth, amenorrhoea, and azoospermia [10, 19, 80, 84, 85].

Recently, new phenolics in different plant parts (leaves, stems, seeds, and petals), including flavonoids and phenolic acids, were reported [10, 19, 82]. In addition, a phytochemical study concerning several classes of metabolites was performed and bioactivity was assessed [81–84]. The high antioxidant potential of *C. roseus* was demonstrated *in vitro* using the radicals DPPH, superoxide, and nitric oxide [19, 81].

2.7. Cyclopia genistoides (L.) Vent. (Fabaceae)—Honeybush. *Cyclopia genistoides* is an indigenous herbal tea to South Africa and considered as a health food. Traditionally, the leafy shoots and flowers were fermented and dried to prepare tea. It has also been used since early times for its direct positive effects on the urinary system and is valued as a stomachic that aids weak digestion without affecting the heart. It is a drink that is mainly used as a tea substitute because it contains no harmful substances such as caffeine. It is one of the few indigenous South African plants that made the transition from the wild to a commercial product during the past 100 years. Research activities during the past 20 years have been geared towards propagation, production, genetic improvement, processing, composition, and the potential for value adding [19, 25–31, 45].

A decoction of honeybush was used as a restorative and as an expectorant in chronic catarrh and pulmonary tuberculosis [25–31, 86]. Drinking an infusion of honeybush apparently also increases the appetite, but no indication is given of the specific species [87]. According to Marloth [88], honeybush was praised by many colonists as being wholesome, valuing it as a stomachic that aids weak digestion without producing any serious stimulating effects on the heart. It also alleviates heartburn and nausea [25–31]. Anecdotal evidence suggests that it stimulates milk production in breast-feeding women and treats colic in babies [89].

Modern use of honeybush is prepared as an infusion and at times taken together with rooibos. The tea bags usually contain a larger percentage of rooibos than honeybush. Parts of other indigenous South African plants and fruits mixed with honeybush include dried buchu leaves, pieces of African potato (*Hypoxis hemerocallidea*) corms, and dried marula (*Sclerocarya birrea*) fruit. The ready-to-drink honeybush iced tea market is not developed to the same extent as that of rooibos, while honeybush "espresso," amongst others, has not been tried [10, 19].

Honeybush is well known as caffeine-free, low tannin, aromatic herbal tea with a wealth of polyphenolic compounds associated with its health-promoting properties. The exact biologically active phytochemicals from honeybush are unknown, but the beneficial effects have been justified based on phenolic compounds. The major compounds, present in all species analyzed to date, are the xanthones, mangiferin and isomangiferin, and the flavanone, hesperidin. Recently, two benzophenone derivatives 3-*C*-β-glucosides of maclurin and iriflophenone were isolated for the first time from *C. genistoides* and were tested for pro-apoptotic activity toward synovial fibroblasts isolated from rheumatoid arthritis patients. The strongest proapoptotic activity was obtained

for isomangiferin and iriflophenone 3-C-β-glucoside, which were not previously evaluated as potential antiarthritic agents. Proapoptotic effects were recorded for mangiferin and hesperidin, which are major polyphenols in all commercially used honeybush plants. Recently, *C. genistoides* has attracted much attention for the production of an antioxidant product high in mangiferin content. The latter and its sustainability make *C. genistoides* an attractive source of mangiferin. Other potential applications are for the prevention of skin cancer, alleviation of menopausal symptoms, and lowering of blood glucose levels [10, 19, 45, 90].

2.8. Harpagophytum procumbens (Burch.) DC. (Pedaliaceae)—Devil's Claw. *Harpagophytum procumbens* is native to the red sand areas in the Transvaal of South Africa, Botswana, and Namibia. It has spread throughout the Kalahari and Savannah desert regions. The indigenous San and Khoi peoples of Southern Africa have used Devil's Claw medicinally for centuries, if not millennia [8, 19, 91]. *Harpagophytum procumbens* has an ancient history of multiple indigenous uses and is one of the most highly commercialized indigenous traditional medicines from Africa, with bulk exports mainly to Europe where it is made into a large number of health products such as teas, tablets, capsules, and topical gels and patches [92].

Traditional uses recorded include allergies, analgesia, anorexia, antiarrhythmic, antidiabetic, antiphlogistic, antipyretic, appetite stimulant, arteriosclerosis, bitter tonic, blood diseases, boils (topical), childbirth difficulties, choleretic, diuretic, climacteric (change of life) problems, dysmenorrhea, dyspepsia, edema, fever, fibromyalgia, fibrositis, gastrointestinal disorders, headache, heartburn, indigestion, liver and gall bladder tonic, malaria, migraines, myalgia, neuralgia, nicotine poisoning, sedative, skin cancer (topical), skin ulcers (topical), sores (topical), tendonitis, urinary tract infections, and vulnerary for skin injuries. The major clinical uses for Devil's claw are as an anti-inflammatory and analgesic in joint diseases, back pain, and headache. Evidence from scientific studies in animals and humans has resulted in widespread use of standardized Devil's claw as a mild analgesic for joint pain in Europe [8, 12, 19, 91–95].

Chemical constituents according to the published literature include potentially (co)active chemical constituents: iridoid glycosides (2.2% total weight) harpagoside (0.5–1.6%), 8-p-coumaroylharpagide, 8-feruloyl-harpagide, 8-cinnamoylmyoporoside, pagoside, acteoside, isoacteoside, 6′-O-acetylacteoside, 2,6-diacetylacteoside, cinnamic acid, caffeic acid, procumbide, and procumboside. The constituent 6-acetylacteoside, being present in *H. procumbens* and absent in *H. zeyheri*, allows users to distinguish between the two species. Other compounds include flavonoids, fatty acids, aromatic acids, harpagoquinone, stigmasterol, beta-sitosterol, triterpenes, sugars (over 50%), and gum resins [8, 12, 19, 91–95]. Harpagoside isolated from *H. procumbens* varies within the plant and is the highest in the secondary tubers, with lower levels in the primary roots. The flowers, stems, and leaves appear to be devoid of active compounds. Iridoid glycosides isolated from *H. procumbens* tend to show dose-dependent anti-inflammatory and analgesic effects equivalent to phenylbutazone; they are apparently inactivated by gastric acids. Harpagoside is the most effective when given parenterally and loses potency markedly when given by mouth; enteric coated preparations might maintain efficacy despite exposure to gastric acids. Harpagoside has also been reported to inhibit arachidonic acid metabolism through both cyclooxygenase and lipoxygenase pathways [9, 91–96].

There are varying and contradictory opinions regarding the anti-inflammatory and analgesic effects of Devil's claw in the treatment of arthritic diseases and low back pain. The controversy revolves around the active constituent of Devil's claw and its mechanism of action, as it appears to be different than that of nonsteroidal anti-inflammatory drugs (NSAID). The evidence from scientific studies in animals and humans has resulted in widespread use of standardized Devil's claw as a mild analgesic for joint pain in Europe [8, 12, 19, 91–96].

Several clinical studies have been performed to determine the effectiveness of *H. procumbens* for its use as anti-inflammatory, general analgesic (commonly for lower back pain), and anti-rheumatic agent. To determine the effectiveness on lower back pain, *Harpagophytum* extract WS1351 was administered in two daily doses of 600 and 1200 mg containing 50 and 100 mg of harpagoside, respectively, and compared to placebo. This randomized double-blind study took place over 4 weeks and subjects ($n = 197$) with chronic susceptibility to back pain and current exacerbations with intense pain were included. Out of 183 subjects that completed the trial, six in the 600 mg and 10 in the 1200 mg were reported "pain-free" without using Tramadol (rescue pain medication). However, data analyses suggested that the 600 mg group reaped more benefits where less severe pain and no radiation or neurological deficit was present. The patients with more severe pain tended to use more Tramadol but not to the maximum permitted dose [92].

2.9. Momordica charantia Linn. (Cucurbitaceae)—Bitter Melon. *Momordica charantia*, also known as bitter melon, is a tropical vegetable grown throughout Africa. The leaf may be made into a tea called "cerassie," and the juice, extracted from the various plant parts (fruit pulp, seeds, leaves, and whole plant), is very common folklore remedy for diabetes [2]. *M. charantia* has a long history of use as a folklore hypoglycemic agent where the plant extract has been referred to as vegetable insulin [97].

Several active compounds have been isolated from *M. charantia*, and some mechanistic studies have been done [98–101]. Khanna et al. [102] have reported the isolation from fruits, seeds, and tissue culture of seedlings, of "polypeptide-p," a 17-amino acid, 166-residue polypeptide which did not cross-react in an immunoassay for bovine insulin. Galactose binding lectin with a molecular weight of 124,000 isolated from the seeds of *M. charantia* has been evaluated for its antilipolytic and lipogenic activities in isolated rat adipocytes and found comparable to insulin [103, 104]. Extracts of fruit pulp, seed, leaves, and whole plant of *M. charantia* have shown hypoglycemic effect in various animal models [103, 104]. Karunanayake et al. [105] found a significant improvement in glucose tolerance and hyperglycemia when

the fruit juice of bitter melon was administered orally to rats. Fresh as well as freeze-dried *M. charantia* was found to improve glucose tolerance significantly in normal rats and noninsulin dependent diabetics (NIDDM) [105–108]. It was hypothesized that *M. charantia* fruit contains more than one type of hypoglycemic component. These may include an active principle called "charantin," a homogenous mixture of β-sitosterol-glucoside and 2-5-stigmatadien-3-β-ol-glucoside that can produce a hypoglycemic effect in normal rabbits [109]. Shibib et al. [110] in a study administered the ethanolic extract of the fruit of bitter melon to STZ diabetic rats orally at a dose equivalent to 200 mg extract per kg body weight. Ninety minutes after the administration, they found that blood glucose levels had been reduced by 22%. The glucogenic enzymes-glucose-6-phosphatase and fructose 1, 6-bisphosphatase in the liver were also depressed. Further evidence for a beneficial chronic effect is that an improvement in both glucose tolerance and fasting blood glucose levels was observed in 8 NIDDM patients following 7 weeks of daily consumption of powered *M. charantia* fruit [97]. Although some authors have indicated that the effect of *M. charantia* is not associated with an increase in circulating insulin, Welihinda et al. [111] and Welihinda and Karunanayake [112] demonstrated that an aqueous extract from the fruit of *M. charantia* was a potent stimulator of insulin release from β-cell-rich pancreatic islets isolated from obese-hyperglycaemic mice. Recently, Matsuura et al. [113] have isolated an α-glucosidase inhibitor from *M. charantia* seeds which can be a potential drug therapy for postprandial hyperglycaemia (PPHG). However, Dubey et al. [104] found that the aqueous, methanolic, and saline extracts of *M. charantia* produced a significant hypoglycaemic effect in rats. In addition, the methanol and saline extracts also prevented adrenaline-induced hyperglycaemia.

When tested on laboratory animals, bitter melon has shown hypoglycaemic as well as antihyperglycaemic activities. Polypeptide-p isolated from fruit, seeds, and tissue of bitter melon showed potent hypoglycaemic effects when administered subcutaneously to gerbils, langurs, and humans. The aqueous extracts of *M. charantia* improved oral glucose tolerance test (OGTT) after 8 h in normal mice and reduced hyperglycaemia by 50% after 5 h in STZ diabetic mice. In addition, chronic oral administration of extract to normal mice for 13 days improved OGTT while no significant effect was seen on plasma insulin levels. We recently reported that *M. charantia* fruit extracts have a direct impact on transport of glucose *in vitro* [99, 114–117].

2.10. Pelargonium sidoides DC. (Geraniaceae)—Umckaloabo. *Pelargonium sidoides* is native to the coastal regions of South Africa, and available ethnobotanical information shows that the tuberous *P. sidoides* is an important traditional medicine with a rich ethnobotanical history [19].

P. sidoides root extract EPs 7630, also known as Umckaloabo, is a herbal remedy thought to be effective in the treatment of acute respiratory infections. There are numerous studies about *P. sidoides* and respiratory tract infections [118, 119]. These studies showed that *P. sidoides* may be effective in alleviating symptoms of acute rhinosinusitis and the common cold in adults, but doubt exists. It may be effective in relieving symptoms of acute bronchitis in adults and children and sinusitis in adults [118]. EPs 7630 significantly reduced bronchitis symptom scores in patients with acute bronchitis by day 7 [119]. No serious adverse events were reported. EPs 7630 has a positive effect on phagocytosis, oxidative burst, and intracellular killing of cells [120–125]. *P. sidoides* extract modulates the production of secretory immunoglobulin A in saliva, both interleukin-15 and interleukin-6 in serum, and interleukin-15 in the nasal mucosa [19, 126].

In one research, *P. sidoides* was documented to represent an effective treatment against common cold. It was reported to significantly reduce the severity of symptoms and shortens the duration of the common cold compared with placebo. Because of these effects, the authors have aimed at establishing whether or not *P. sidoides* could affect the asthma attack frequency after upper respiratory tract viral infection. Results for some 20 clinical trials have been published, 7 of which were observational studies and the remaining 13 were randomized, double-blind, and placebo-controlled. For 6 of those 13 trials, only preliminary results have been published. All trials have been carried out using EPs 7630 in liquid or solid forms. The data derived from these trials has been evaluated in 2 reviews [19, 118, 119].

Antibacterial activity of extracts and isolated constituents of *P. sidoides* was evaluated by Kayser [127] against three gram-positive and five gram-negative bacteria. Most compounds exhibited antibacterial activities. Further investigations by Lewu et al. [128] have supported these findings. EPs 7630 has been found to show a synergistic indirect antibacterial effect in group A-streptococci (GAS) through inhibition of bacterial adhesion to human epithelial cells as well as induction of bacterial adhesion to buccal epithelial cells [8, 129]. Wittschier et al. [130] and Beil and Kilian [131] confirmed the antiadhesive effect of EPs 7630 for *Helicobacter pylori* growth and adhesion to gastric epithelial cells. Umckaloabo has been documented to significantly stimulate phagocytosis, oxidative burst, and intracellular killing whichwas also enhanced [120–125]. It was proposed that modulation of epithelial-cell bacteria interaction through EPs 7630 may protect mucous membranes from microorganisms evading host defense mechanisms. These findings tend to provide a rationale for the treatment of upper respiratory tract infections with EPs 7630 [131, 132].

Taylor et al. [133] have recently established the antimycobacterial activity for hexane extracts of roots of *P. reniforme* and *P. sidoides*. Gödecke et al. [134] have reported no significant effect on the bacterial growth of two strains of mycobacteria by extracts and fractions of *P. sidoides*. An antitubercular effect may thus be achieved indirectly by stimulation of immune response. This assumption was supported by Mativandlela et al. [135] as none of the compounds isolated from *P. sidoides* showed any significant activity against *M. tuberculosis*.

Kayser et al. [136] have investigated into extracts and isolated constituents of *P. sidoides* for their effects on nonspecific immune functions in various bioassays. They found indirect activity, possibly through activation of macrophage functions. Activation was confirmed through the presence

of tumor necrosis factor (TNF-alpha) and inorganic nitric oxides (iNO). Kolodziej [137, 138] also observed TNF-inducing potencies for EPs 7630 as well as interferon-like activities. Koch et al. [139] observed interferon- (IFN-) beta production increased and natural killer cell mediated cytotoxicity enhanced in MG-63 human osteosarcoma cells preincubated with Umckaloabo [19, 139]. Kolodziej [137, 138] investigated polyphenol-containing extracts of *P. sidoides* and simple phenols, flavan-3-ols, proanthocyanidins, and hydrolysable tannins for gene expressions (iNOS, IL-1, IL-10, IL-12, IL-18, TNF-alpha, and IFN-alpha/gamma). All extracts and compounds were capable of enhancing the iNOS and cytokine mRNA levels in parasitised cells.

3. Discussion

Medicinal plants are an integral part of the African healthcare system since time immemorial. Interest in traditional medicine can be explained by the fact that it is a fundamental part of the culture of the people who use it and also due to the economic challenge: on one side, the pharmaceutical drugs are not accessible to the poor and on the other side, the richness and diversity of the fauna and flora of Africa are an inexhaustible source of therapies for panoply of ailments [140]. Nonetheless, there is still a paucity of clinical evidence to show that they are effective and safe for humans. Without this information, users of traditional medicinal plants in Africa and elsewhere remain skeptical about the value of such therapies. This denies people the freedom to choose plants that are potentially less costly and are more accessible. Another issue concerning the use of botanical remedies is the need to understand the safety of these therapies. For these reasons, information about efficacy and safety of traditional medicines is urgently required. The present paper has endeavoured to overview just a few common medicinal plants from the African continent which have short- as well as long-term potential to be developed as future phytopharmaceuticals to treat and/or manage panoply of infectious and chronic conditions. Within the framework of enhancing the significance of traditional African medicinal plants, aspects such as traditional use, phytochemical composition, and *in vitro*, *in vivo,* and clinical studies pertaining to the use of these plants have been explored.

During the last few decades, it has become evident that there exists a plethora of plants with medicinal potential and it is increasingly being accepted that the African traditional medicinal plants might offer potential template molecules in the drug discovery process. Many of the plants presented here show very promising medicinal properties thus warranting further clinical investigations. Nonetheless, only few of them have robust scientific and clinical proofs and with a significant niche market (e.g., *Aloe ferox*, *Artemisia afra*, *Aspalathus linearis*, *Centella asiatica*, and *Pelargonium sidoides*) and a lot more have yet to be explored and proved before reaching the global market [7, 8, 12, 19].

In the light of modern science, significant efforts should be geared to identify and characterize the bioactive constituents from these plants. Indeed, the discovery of therapeutic compounds from traditional medicinal plant remedies remains a medically and potentially challenging task. For adventure in such an attempt, highly reproducible and robust innovative bioassays are needed in view of our limited understanding of the multifactorial pathogenicity of diseases. Innovative strategies to improve the process of plant collection are needed, especially with the legal and political issues surrounding benefit-sharing agreements. Since drug discovery from medicinal plants has traditionally been so time-consuming, it is also of uttermost importance for investigators to embark and devise new automated bioassays with special emphasis on high throughput procedures that can screen, isolate, and process data from an array of phytochemicals within shorter time lapse for product development. Additionally, these procedures should also attempt to rule out false positive hits and dereplication methods to remove nuisance compounds [7].

Nonetheless, despite continuous comprehensive and mechanism-orientated evaluation of medicinal plants from the African flora, there is still a dearth of literature coming from the last decade's investigations addressing procedures to be adopted for quality assurance, authentication, and standardization of crude plant products. Appropriate standardization could be achieved via proper management of raw material, extraction procedures, and final product formulation. Without effective quality control, consistency and market value of the herbal product may be compromised. Indeed, one of the main constraints to the growth of a modern African phytomedicine industry has also been identified as the lack of proper validation of traditional knowledge and also the lack of technical specifications and quality control standards. This makes it extremely difficult for buyers, whether national or international, to evaluate the safety and efficacy of plants and extracts, or compare batches of products from different places or from year to year. This is in marked contrast with Europe and Asia where traditional methods and formulations have been recorded and evaluated both at the local and national levels. This would also tend to justify why the level of trade of phytomedicines in Asia and Europe is blooming more than those in Africa [7].

It is also imperative that potential risk factors, for example, the contamination of medicinal plant products with heavy metals from African traditional medicine products, be addressed and that regulatory guidelines are not only carefully developed but also enforced. Controlled growth (under GACP) and processing environments (under Good Manufacturing Practice) need to ensure that contamination of medicinal plant material is kept to a minimum. For the medicinal plant industry, cultivated plant material is preferred as it is easier to control the supply chain plus contamination is nominal [141]. On the other hand, proper identification of a medicinal plant material is fundamental to the quality control process; it must be established unmistakably that the source of the plant material is genuine. Following this, microbial contamination (fungal, bacterial, and any potential human pathogens) must be checked during the stages of processing of the material. Chemical, pharmacological, and toxicological evaluations, conducted according to the principles of Good Laboratory Practices (GLPs), will certify the bioactive properties of the material undergoing processing.

These tests also are often the predictors of safety of the products manufactured. Clinical safety and efficacy will need to be established through exhaustive and usually lengthy trials during the early stages of the development of a therapeutic agent. After that, so long as the standard operating procedures are adhered to, then the unit dosage forms produced will be considered safe. Notwithstanding this, quality assurance procedures must be instituted so that the products coming from the factory are of good quality, safety, and efficacy [142]. To this effect, during the development stage, product standardization, quality control and assurance, double-blind, placebo-controlled, and randomized clinical controlled trials using standardized products or products containing pure plant extracts are essential components that need to be perfected in order to translate the potential of African botanicals into a reality for human to benefit [7, 142].

4. Conclusions

It is evident from the literature that there is currently a renewed interest in African-plant-based medicines in the prevention and cure of various pathologies. Medicinal plants still play an important role in healthcare system in African countries. Nonetheless, there are still many major challenges that need to be overcome and addressed for its full potential to be realized as the effective treatment of diseases with plant products has not been validated thoroughly with robust scientific criteria to compete with existing conventional therapies. Additionally, other issues that need to be addressed are that of access and benefit sharing following the Nagoya agreement. Local laws need to be TRIPS compliant if trade of African herbal products is to increase, and, at the same time, issues of sustainable use and development of plant products need to be addressed.

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Review Article

Mushrooms and Truffles: Historical Biofactories for Complementary Medicine in Africa and in the Middle East

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The ethnopharmaceutical approach is important for the discovery and development of natural product research and requires a deep understanding not only of biometabolites discovery and profiling but also of cultural and social science. For millennia, epigeous macrofungi (mushrooms) and hypogeous macrofungi (truffles) were considered as precious food in many cultures based on their high nutritional value and characterized pleasant aroma. In African and Middle Eastern cultures, macrofungi have long history as high nutritional food and were widely applied in folk medicine. The purpose of this review is to summarize the available information related to the nutritional and medicinal value of African and Middle Eastern macrofungi and to highlight their application in complementary folk medicine in this part of the world.

1. Introduction

From early stages of civilization, desert macrofungi in forms of mushrooms and truffles have been used as food and medicine. Originally, these types of organisms were associated with Mediterranean region and were first recorded as poem in Egyptian temples as follows: "Without leaves, without buds, without flowers: yet they from fruit; as a food, as a tonic, as a medicine: the entire creation is precious." Thus, macrofungi were considered as food and medicine for royalty, and that no normal citizens were allowed to consume this precious food. During Greek and Roman eras, they were imported from Libya and sold in southern part of the European continent [1]. In the southern part of African continent, the nomadic people of Kalahari Desert used truffles for millennia [2].

Mushrooms are visible to the naked eye as they grow above the earth, whereas truffles grow underground in depth between 5 and 10 cm. Truffles are usually collected by specialists who have special skills and experience to explore this type of flora. Sometimes, truffle collectors use some animals such as pigs and dogs to discover this type of underground fungus. This is based on their high sensitivity to the characteristic truffle volatile compounds. Traditionally, mushrooms and truffles are taken as type of precious food and consumed either raw or cooked. In addition, they have been also applied as main component of folk medicine. This was based on the fact that they are rich source for proteins, amino acids, fatty acids, fiber, minerals, vitamins, terpenoids, sterols, flavor compounds, and carbohydrates as reported by many authors [3–5].

In general, not all types of macrofungi are able to grow in the harsh environmental conditions of desert. The term "Desert Macrofungi: Mushrooms and Truffles" is related to the nature and distribution of those species which can grow under arid and semiarid conditions. Thus, the geographical distribution of desert truffles in Africa and Middle East is related to countries around the Mediterranean such as Morocco, Algeria, Tunisia, Libya, Egypt, and Israel in addition to the countries of the Arabian Peninsula such as Jordan, Syria, Saudi Arabia, Iraq, Bahrain, and Kuwait. However, some types of desert truffles were also found in South Africa

and Botswana [6]. Generally, the growth of desert truffles requires an annual rainfall range between 50 and 380 mm. In North Africa, good yields of truffle are usually obtained if the rainfalls range between 70 to 120 mm. In addition, the time, quantity, and distribution of the rainfall play an important role in the quality of desert macrofungus growth. For example, to obtain good truffles in North African and Middle Eastern regions, it needs to get rainfall no later than the beginning of December whereas, in southern Europe it should not be later than the beginning of October [7].

For centuries, it was proposed that most of the wild macrofungi are not cultivable. However, with the increased knowledge of mushroom and truffles physiology, nowadays, it is possible to cultivate many types of macrofungi. Mushrooms were successfully cultivated in green houses and in submerged culture fermentation. Whereas, based on the symbiotic behavior of truffles as typical ectomycorrhiza, they were cultivated in soil with their host plant in truffle green houses in semiarid area [6, 7]. With the rapid growth of bioprocess technology industries, it was possible to cultivate many macrofungi in submerged culture under fully controlled conditions to produce the desired biotherapeutic compounds in high concentrations, in shorter production time, and under fully sterile conditions according to cGMP [8–10].

This review outlines the current status of knowledge on the macrofungi bioactive compounds and their applications in complementary and alternative medicine in different African and Middle Eastern cultures.

2. Type and Identification

Different types of desert epigeous macrofungi (mushrooms) and hypogeous macrofungi (truffles) are considered as natural flora in the Middle East and the African desert. Mushrooms are characterized by their distinguished structure of stalk and their fragile and soft feature. Truffles have no stalk and no gills with firm, dense, and woody feature. The name truffles/*terfezia* comes from the word "Terfass," which is an Arabic word describing hypogeous desert fungus. Truffles are also known locally in North Africa and Arabian Peninsula under other names such as "Al-Kamaa" or "Al-Fag'a." Taxonomically, truffles are ascomycetes fungi belonging to the genus *Tuber*, the family Tuberaceae, and the order Pezizales [11] and are distributed in six pezizalean families: Glaziellaceae, Discinaceae, Morchellaceae, Helvellaceae, Tuberaceae, Pezizaceae, and Pyronemataceae [12]. In general, most of known desert truffles species belong to genera such as *Terfezia, Delastreopsis*, *Balstonia, Delastria, Leucangium, Mattirolomyces, Phaeangium Picoa, Tirmania*, and *Tuber* [6]. Ecologically, desert truffles are symbiotic microorganisms and they establish ectomycorrhizal symbiotic relationship with species of *Helianthemum* genus. Therefore, the distribution of desert truffles is limited not only by the environmental conditions but also by the availability of the host plant. For example, in Arabian Peninsula, only three types of truffles belonging to *Terfezia*, the two black-dark brown colored truffles *Terfezia claveryi* and *Terfezia boudieri* (known locally as Ikhlasi), and the white-cream colored truffle belonging to *Tirmania nivea* (known locally as Zubaidi) are available in this region [13]. In addition, another species of truffle *Phaeangium lefebvrei* is commonly known as bird truffle "Faga Al toyoor or Hopper" and is mainly consumed by birds. In North Africa, in addition to the previous local names, many other names are also given for truffles such as "Nabat Al Radh, asqal, Bidat El Ardh and Banat Ober" [14].

Mushrooms spread in wide area in Africa and Middle East. The most common types of mushrooms belong to *Agaricus* and *Pleurotus* sp. (Basidiomycetes). In North Africa, different types of *Pleurotus* sp. were found only for few days after rainy season. Based on its morphological structure and physiology, mushrooms are more sensitive to high temperature and dry conditions compared to truffles. The tropical and subtropical regions of Africa are characterized by higher mushroom diversity compared to North Africa. Table 1 shows some examples of different types of mushrooms and truffles in Africa and Middle East.

3. Nutritional Value

Desert macrofungi are rich source of different types of essential nutrients, and thus their nutritional value and chemical profile were studied and reviewed by many authors [15]. The chemical analysis of desert truffles showed that the dry matter is composed of up to 60% carbohydrates, 20–27% protein, 3–7.5% fat (unsaturated and saturated fatty acids), 7–13% fiber, and 2–5% ascorbic acid [12]. Another study for chemical profiling of three Iraqi truffles (*Terfezia claveryi, Tirmania nivea*, and *Tirmania pinoyi*) showed that the carbohydrate concentration in dry matter ranged between 16.6 and 24.8%, protein content ranged from 8.1 to 13.8%, phosphorus from 9.7–25.5%, and ash 4.9–5.9% [16]. Another research showed also that the chemical composition of truffle is highly strain specific and the white desert truffle *Tirmania nivea* (Zubaidi) was higher in protein, fat, and crude fiber content compared to other two types of desert truffles belonging to black truffle group (Gibeah and Kholeissi) [17]. However, the same study showed also that all essential amino acids were present in all three truffles including the sulphur amino acids (methionine, cysteine, tryptophan, and lysine) which are usually the limiting amino acids in many foods of plant origin. In addition, different studies demonstrated also that truffles are rich source of essential minerals such as Si, K, Na, Ca, Mg, Mn, Fe, Al, Cu, and Zn [15].

Unlike truffles, the dry content of mushrooms is usually in the range between 60 and 140 g/kg. In most types of mushrooms, carbohydrates and crude proteins are the main two components. The composition of mushroom fruit bodies is very rich with carbohydrates, and its concentration is ranging between 20 up to more than 70% of the dry weight and is highly dependent on mushroom strain. Glucose, mannitol, and trehalose, their derivatives and oligosaccharides, are the main polysaccharides and share very low percentage in fruit body dry weight. Unlike plants which store polysaccharide in the form of starch, mushrooms store polysaccharides in the form of glycogen and usually contribute to about 5–10% of dry matter [5]. Chitin, a water insoluble polysaccharide,

Table 1: Some examples of different types of wild macrofungi in Africa and in the Middle East region.

	Countries	References
Type of truffle		
Terfezia boudieri Chatin	Libya	[72]
Terfezia claveryi	Saudi Arabia, Bahrain, Iraq, Egypt, and Jordan	[29, 45]
Terfezia pfeilii	South Africa	[86]
Terfezia sp.	Tunisia	[87]
Tirmania nivea	Saudi Arabia, Morocco, Bahrain, Egypt, and Kuwait	[13, 16, 73, 88]
Phaeangium lefebvrei	Bahrain, Saudi Arabia, Egypt, and Kuwait	[14]
Choiromyces echinulatus	South Africa	[86]
(Eremiomyces echinulatus)		[82]
Picoa juniper	Tunisia	[89]
Picoa lefebvrei	Tunisia	[89]
Kalaharituber pfeilii	South Africa, Botswana	[82]
Types of mushrooms		
Pleurotus sp.	Almost in all African and Middle Eastern countries	[51]
Agaricus sp.	Almost in all African and Middle Eastern countries	
Lentinus sp.	Cameroon, Nigeria	[90]
Russula sp.		
Cantharellus sp.	Madagascar, Zimbabwe	[91, 92]
Afroboletus luteolus		
Termitomyces sp.		[93]
Lactarius sp.	Zambia, Zimbabwe, Mozambique, Tanzania, Zaire, and Benin	[94]
Cantharellus sp.		[95]
Amanita zambiana		
Schizophyllum commune	Ethiopia	[96]
Chlorophyllum sp.	Burkina Faso	[97]
Phlebopus sp.		

contributes to up to 80–90% of mushroom cell wall in addition to other components such as hemicelluloses and pectin [18]. Mushrooms contain also high content of insoluble fiber which also increases its nutritional value. On the other hand, mushrooms are very rich in proteins and usually comprise about 30% of dry weight. Protein distribution is usually changeable during the fungal development. It was reported that, for the widely distributed oyster mushroom (*Pleurotus ostreatus*), the highest crude protein content of about 36% with highest digestibility (92%) is mainly found in fungal cap of diameter ranging between 5 and 8 cm [19]. In addition to the high fungal protein content, the proportion of essential amino acids makes it of higher nutritional value compared to plant proteins [5]. The mushroom lipid content ranges between 2 and 6% of dry weight. The polyunsaturated linoleic acid, the monounsaturated oleic acid, and the saturated palmitic acids are the main components of mushroom fatty acids. Other branched chain acids and hydroxyl fatty acids are also present in some mushrooms but in very low concentrations [20, 21]. Mushrooms also include ash (5–12% of dry matter) and are rich in essential elements such as sodium, potassium, calcium, magnesium, phosphorus, and sulfur. Moreover, mushrooms also include many essential trace elements such as manganese, cupper, and selenium. The presence of chromium and selenium in acceptable concentrations (few mg per kg dry matter) increases the nutritional value of mushrooms and is considered as potential source of organic selenium/chromium in diets and food supplements [5].

4. Volatile Organic Compounds

In general, macrofungi are characterized by their ability to produce wide range of volatile compounds which gives the characteristic flavor of this group of organisms. The characteristic aroma compounds of fruiting bodies are often used for strain nomenclature in both Latin and local names to indicate special aromatic characteristics. For example, attributes like *butyrace-* (butter like), *odor-losm-* (fragrant), *delicat-* (delicious), *olid-* (ambrosial, *suav-* (sweet), and *nidoros-* (pungent) point out certain tastes and aromas [22].

Truffles are typical ectomycorrhizal fungi and thus must grow in association with host plant. The hypogeous fruiting bodies of this type of organisms are characterized by their strong aroma which helps the truffle hunters to find them in the soil. This complex aroma comes from easily evaporated low molecular weight carbon compounds

which have common name as volatile organic compounds (VOCs). These compounds play crucial ecological role in the recognition between the fungus and the host plant and also regulate the symbiosis process. In addition, these compounds play another role in the interaction between the truffles and nonhost plant as well. Nowadays, more than 200 VOCs and many nonvolatile compounds have been isolated and identified from truffles fruiting bodies. Truffles VOCs include fatty acids, terpenoids, aromatic compounds, and sulfur containing compounds [23]. On the other hand, mushroom volatile flavors and VOCs are one of the fungal characteristics which sometimes determine their commercial value. In general, the flavor and special aroma of certain mushrooms are usually a combination of different VOCs. The chemical profile of volatile compounds of *P. ostreatus* includes C_8 compounds such as oct-1-ene-3-ol, octan-3-ol, octan-3-one, octanal, oct-1-en-3-one, and octan-1-ol in addition to benzaldehyde (almond odor), benzyl alcohol (sweet spicy odor), and monoterpenes like linalool and linalool oxide. These all together make the characteristic aroma and pleasant flavor of this mushroom [24]. In other mushrooms like *Termitomyces schimperi*, which is common fungus in many African countries, the VOCs of fresh fruiting bodies include more than twenty-four compounds. They include oct-1-en-3-ol, 2-phenylethanol, and hexanal. It was also interesting that these VOCs were almost the same in both of caps and stems of this fungus [25].

However, these characteristic fungal VOCs are important during fungal life cycle, not only to increase their market value but also to contribute to the nutritional and medicinal values of macrofungi.

5. Functional Chemical Compounds of Health Value

5.1. Antioxidants. Macrofungi are well known to contain different bioactive polyphenolic compounds. These compounds act as effective antioxidants based on their excellent ability to scavenge free radicals and act as reducing agents. Thus, it was proposed by many authors that there are strong correlations between the antioxidant activities of certain type of fungi and the type and concentration of polyphenolic compounds. Different groups of desert truffles and mushrooms showed strong antioxidant activities based on their high polyphenolic and ergosterol contents [13]. In the study of Villares et al. [26], different types of bioactive organic compounds showing antioxidant activities were isolated from *Tuber* sp. These include, ergosterol such as ergosteryl ester, wide range of phenolic acids such as gallic, homogentisic, protocatechuic, *p*-hydroxybenzoic, and *o*- and *p*-coumaric acids, and other phenolic derivatives such as 3,4-dihydroxybenzaldehyde. The study of Al-Laith [13] showed also that the famous white desert truffle *T. nivea* showed high antioxidant activities. It was claimed that the fungal antioxidant capacity is attributed to the presence of various chemicals such as ascorbic acid, carotenoids, esterified phenolics, and free- and nonflavonoid phenolics and flavonoids. Another research showed that the small truffle *Picoa lefebvrei* is also among the most attractive mushrooms in folk medicine based on its high antioxidant properties [27].

5.2. Antimicrobial Activity. Since the early 20th century, fungi are known for their highly potent antimicrobial secondary metabolites. However, the antibacterial and antiviral activities of desert truffles were first studied in 1980s by Al-Marzooky [28] who investigated the *in vitro* biological activities of all aqueous polar and nonpolar extracts of *T. claveryi*. This extract exhibited good wide spectral antimicrobial activities especially against the trachoma causing disease *Chlamydia trachomatis*, stomach ulcer, and open cut. Another research demonstrated that the use of aqueous extract of *Terfezia claveryi* inhibited the growth of pathogenic bacteria such as *Staphylococcus aureus* [29] and *Pseudomonas aeruginosa* [30]. The antimicrobial activity of *Terfezia* was due to the production of small molecular weight peptide antibiotics [29]. In addition to the wide spectrum antibacterial activities, *Terfezia* extract possessed also antiviral activities [16]. Another research done by Dib-Bellahouel and Fortas [31] showed also that the ethyl acetate extract of *Tirmania pinoyi* exhibited potent antimicrobial activities against the G+ve bacteria *B. subtilis* and *S. aureus.* These all together make mushrooms and truffle important candidates in complementary medicine [32].

5.3. Immunomodulators and Antitumors. Immunomodulators or Biological Response Modifiers (BRMs) are compounds capable of interacting with the immune system to upregulate or downregulate specific aspects of the host response. Some of these compounds exhibiting also anticancer activities were isolated from different types of macrofungi [8, 33]. Different types of BRM were applied as immunotherapeutic agents and showed significant activity as potent anticancer compounds in both *in vitro* and *in vivo* models [34–36]. Of the two main types of macrofungi, mushrooms are a common source of producing this type of compounds naturally. BRMs were isolated from different mushrooms parts such as fruiting bodies, stalk, spores, and mycelium in addition to fermentation broth when cultivated in submerged culture. In some research, BRMs were applied in combination with conventional chemo- and radiotherapy during cancer treatment to increase their efficiency. In general, according to their chemical structure, mushroom BRMs were classified into four main categories: lectins, terpenoids, polysaccharides and their peptides, and fungal immunomodulator proteins (Fips) [8]. Since the discovery of the potent anticancer polysaccharide lentinan (derived from *Lentinus edodes)* by Ikekawa and his group in late 1960s, mushrooms polysaccharides became one of the most interesting topics of research for the discovery and development of new anticancer drugs. [37]. Most of the known mushroom bioactive polysaccharides have various branching types of $(1 \rightarrow 3)$- and $(1 \rightarrow 6)$-β-D-glucan and polysaccharide protein complex [38]. However, the biological activity of mushroom polysaccharides is highly dependent on the length and branching of the chain, chain rigidity, and helical conformation [39]. Pleuran (from *Pleurotus ostreatus*), Lentinan (from *Lentinus edodes*), Grifolan (from *Grifola frondosa*),

Krestin-PSK (from *Polystitcus versicolor*), and Scleroglucan (from *Sclerotina sclerotiorum*) are the most famous bioactive polysaccharides from mushroom origin [8, 40–44].

5.4. Other Medical and Cosmeceutical Applications. The medicinal value of African and Middle Eastern macrofungi is not limited to the above mentioned applications. It was also reported that both truffles and mushrooms have also potential applications as hepatoprotective, cholesterol and sugar lowering, and anti-inflammatory agent [15, 32, 38, 42]. For example, the extract of the known desert truffle (*T. claveryi*) showed powerful hepatotoxic activity against the known hepatotoxin carbon tetrachloride [45]. In addition to the known and the well-studied medical applications, different mushroom extracts were applied in cosmetic products such as moisturizers, skin antifade and antiaging agents, skin revitalizers, and whitening cream [46].

6. Therapeutic Values and Ethnomycological Applications

The total number of mushrooms on earth is expected to be 140,000, among which only 10% (about 14,000) is known [47, 48]. It is strongly believed that a large number of the unknown species to be discovered may be present in Africa. Generally, there is little or no information about the therapeutic and medicinal uses of these mushrooms in Africa. Primarily, this may be attributed to the fact that this information is scattered in a multitude of sources which are not easily accessible to the international English-speaking community [49]. Secondly, unlike Japan and China, where the knowledge on medicinal applications of edible mushrooms has been documented [50], most of the information on indigenous applications of African mushrooms had been passed orally from one generation to the other [51]. Nowadays, the available knowledge on the medicinal applications of African mushrooms comes from elder people selling those [52] as well as from some ethnobotanical monographs summarizing data for a particular region of the world [53–56].

The variation of climatic conditions within the continent is directly reflected on the prevalence of mushrooms in different geographical regions. Accordingly, medicinal applications may differ not only between different countries, but also between different ethnic groups inhabiting the same country. Mushrooms belonging to species of *Termitomyces, Pleurotus, Lentinus, Lenzites, Trametes, Ganoderma, Pycnoporus, Coriolopsis*, and *Calvatia* have been reported to be used in folk medicine in Nigeria [48, 57, 58]. On the other hand, mushrooms of the species *Termitomyces, Agaricus, Boletus, Pleurotus, Cantharellus, Macrolepiota, Ganoderma*, and *Geastrum* have been reported in Tanzania [59, 60]. Moreover, Kamatenesi-Mugisha and Oryem-Origa [61] have reported that the ethnomycological application of toadstool mushrooms from the family Tricholomataceae has been documented in western Uganda. In Burkina Faso, the application of the mushroom *Parkia biglobosa* has been also reported [62].

Mushrooms have been used in sub-Saharan Africa during the Paleolithic period (7000–9000 B.P.), where their application has been traditionally related to mysticism [57, 63]. Historically, the first reports record the application of mushroom as a hallucinogenic agent by the people of the Yoruba tribe in Nigeria [64]. Additionally, Oso [52] reported that the Yoruba traditional doctors applied a medicinal preparation of *Termitomyces microcarpus* for the treatment of gonorrhea. The preparation was administered orally by pounding a large quantity of the mushroom fruiting bodies with the pulp of the fruit *Cucurbita pepo* Linn., the leaves of *Cassia alata* Linn., and other ingredients. They also used oral administration of *Calvatia cyathiformis* in a ground form with other herbal ingredients to treat a disease known in Yoruba as *Masomaso*. This disease is believed to prevent pregnancy, in which the woman begins to discharge effluvium through her vagina. Additionally, they used *C. cyathiformis* ground with *Daldinia concentrica* as a remedy for leucorrhoea, where the patient woman washes her vagina at predetermined intervals.

Recently, Akpaja et al. [51] and Ayodele et al. [65] reported different ethnomycological applications of mushrooms used by the people of the Igbos in South East and the Igalas in North central Nigeria. They reported that the Igbo and Igala tribes have used *Pleurotus tuberrigium* to overcome headache, stomach pain, fever, cold and constipation; *Lentinus squarrosulus* for treating mumps and heart diseases; *T. microcarpus* for treating gonorrhea; *Calvatia cyathiformis* for treating leucorrhea and bareness; *Ganoderma lucidum* for treating arthritis and neoplasia; *G. resinaceum* to reduce blood sugar level (hypoglycemic) and to overcome liver diseases (hepatoprotector); *G. applanatum* used as an antioxidant and anti diabetes; *Volvariella volvaceae* as an antibiotic and antineoplastic; and *Daldinia concentrica* for treating stomach ulcers and skin diseases.

Mdachi et al. [60] reported that in Tanzania and most African countries, some wild mushrooms have been used in traditional medicine. They also reported that in some rural areas of Tanzania, a mushroom soup is provided to mothers after child delivery to enhance fast recovery, while other mushroom species are used as medicines for stomach and heart diseases. In addition, *Ganoderma* species has been used to treat sick cows, while some puffball mushrooms are traditionally used for wound healing in the Kilimanjaro region of Tanzania.

In 2007, Kamatenesi-Mugisha and Oryem-Origa [61] investigated the effect of medicinal plants used by traditional medical practitioners in inducing labor, namely, uterine contractions, during childbirths in and around Queen Elizabeth Biosphere Reserve in Uganda. They found that native women use toadstool mushroom from the family Tricholomataceae to induce labor during childbirth by enhancing uterine contractions.

Moreover, Beiersmann et al. [62] reported that young mothers in Burkina Faso use mushroom to treat a respiratory distress syndrome, locally known as *Dusukun yelema*, of which 80% are due to malarial acidoses. They apply the ashes of burned mushroom (*Parkia biglobosa*) onto the child's chest. Table 2 summarizes some of the ethnomycological

Table 2: Ethnomycological applications of mushrooms in different African countries.

Mushroom	Country	Ethnomycological applications	Reference
Pleurotus tuber-rigium	Nigeria	Treatment of headache, cold, fever, stomach ache, and constipation	
Lentinus squarrosulus	Nigeria	Treatment of mumps and heart diseases	
Termitomyces microcarpus	Nigeria	Treatment of gonorrhea	
Calvatia cyathiformis	Nigeria	Treatment of leucorrhea, bareness, and hiccups	
Ganoderma lucidum	Nigeria	Treatment of arthritis and neoplasia	
G. resinaceum	Nigeria	Lowering blood sugar level and protecting liver cells	[48]
G. applanatum	Nigeria	Antioxidant and used for lowering blood sugar level, as well as antihypertensive	
Schizophyllum commune	Nigeria	Treatment of diabetes	
Volvariella volvaceae	Nigeria	Antibiotic and antineoplastic	
Auricularia auricular	Nigeria	Treatment of hemorrhoids and hemoptysis	
Daldinia concentrica	Nigeria	Treatment of stomach ulcer and upset, skin disease, and whooping cough and prevention of excessive growth of fetus to ease the delivery	
Polyporus officinalis	Nigeria	Treatment of hernia, cough, and catarrh	
Soup of different wild mushrooms	Tanzania	Promote quick recovery of mothers after childbirth	[56]
Ganoderma spp.	Tanzania	Treatment of sick cows	[60]
Puffball mushrooms	Tanzania	Wound healing	[60]
Termitomyces microcarpus	Tanzania	Health promoter and inducer of breast lactation	[59]
Termitomyces titanicus	Tanzania	Treatment of abdominal pain, stomach ache and ulcers, and constipation	[59]
Toadstool mushroom	Western Uganda	Induce labor during childbirth	[61]
Parkia biglobosa	Burkina Faso	Treat respiratory distress syndrome resulting from malarial acidoses	[62]

applications of mushrooms reported in different African countries.

In a recent study, Tibuhwa [59] explored the dietary, therapeutic, and ethnomycological applications of wild mushrooms in communities living around Ngorongoro and Serengeti National Park in Tanzania. The study aimed at exploring the actual taxonomy knowledge of the Kurya and Masai tribes and at developing a baseline data which can contribute to establishing mushroom traditional uses depository. The study revealed that most of the Kurya tribe people use wild mushrooms as either foodstuff or as tonic. Among the mushrooms used for therapeutic applications, they used *Termitomyces titanicus, T. letestu, T. eurhizus*, and *T. auranticus* for the treatment of different intestinal problems, for example, pain, ulcer, constipation, and stomach ache. *Termitomyces microcarpus* was found to be used as immune boosting agent and is given to sick people to speed up their recovery as well as lactating mothers. The study also revealed that both tribes have different manners in using the same mushroom. Table 3 summarizes some of the ethnomycological knowledge on mushroom utilization by both tribes.

Truffles are hypogeous fruiting bodies of the ascomycetous fungi living symbiotically with soil plant roots [66]. Truffle species have a wide range of host plant species, require a calcareous soil, and have different geographical distribution [67, 68]. They have been found through Europe [69], especially in Italy, France, and Spain and throughout China [70], Australia [71], North Africa and sub-Saharan countries [72, 73], and the Middle East [14, 74].

Truffles are widely appreciated as a costly delicacy as well as for their organoleptic properties, especially aroma [67]. Additionally, truffles have been used to promote health and to prevent and to treat several diseases. They have been reported to possess anti-inflammatory, immunosuppressing, and anticarcinogenic properties [13, 75], antioxidant properties [26, 76], and antimicrobial activities [29, 77, 78].

Truffles have been reported in many African countries [79–81]. Desert truffles have been traditionally used by the native people of the Kalahari in southern Africa for millennia [2, 82], as well as by the Saharan natives [73, 83, 84]. Truffles belonging to the species of *Terfezia* and *Tirmania* have been generally eaten by the native people of north Africa and the Middle East from prehistoric times. The Khoisan people (sometimes called the Bushmen or San) have used truffles from the species of *Kalaharituber, Eremiomyces*, and *Mattirolomyces* [82].

Contrary to mushrooms, less attention has been paid to the indigenous information about medicinal truffles in Africa. The available information comes from some amateurs and foreign tourists' documentation, as well as from knowledge accumulation passed orally from generation to the next [14]. Omer et al. [73] reported that desert truffles (*Tirmania* and *Terfezia* spp.) have been utilized in folk medicine for the treatment of ophthalmic diseases and as aphrodisiac agents. Moreover, the same truffles have been also reported to be used

Table 3: Ethnomycological knowledge on mushrooms utilization by the Kurya and Maasai tribes around Ngorongoro and Serengeti National Park, Tanzania, modified from [59].

Species	Kurya	Masai	Kurya	Masai
Termitomyces microcarpus	Bitoghose	Not known	Food: improve healthy to long-ill people and lactating mothers	Not known
T. titanicus	Lyugu	Ormambuli	Food: tonic for various gastrointestinal problems	Few know it as tonic for various gastrointestinal problems
T. aurantiacus	Nyankobhiti	Ormambuli	Food: tonic for stomach aching	Not known
T. clypeatus	Vihungumururyo	Ormambuli	Food	Not known
T. eurhizus	Amanyegiswa	Ormambuli	Food	Not known
T. le-testui	Lyugu	Ormambuli	Food	Few know it as tonic for various gastrointestinal problems
T. mammiformis	Bitoghose	Ormambuli	Food	Not known
T. umkowaan	Amughu	Ormambuli	Food	Few know it as tonic for various gastrointestinal problems
T. tylerianus	Bitoghose	Ormambuli	Food	Not known
T. striatus	Bitoghose	Ormambuli	Food	Not known
Agaricus campestris	Bitoghose	Ormambuli	Food	Not known
Macrolepiota procera	Binyankorogoto	Not known	Healing wounds	Not known
Ganoderma boninense	Binyankorogoto	Not known	Treat wound and skin infections	Not known
Geastrum saccatum	Uiborinyiti	Not known	Subject bees into anaesthesia state	Subject bees into anaesthesia state
G. triplex	Uiborinyiti	Not known	Subject bees into anaesthesia state	Subject bees into anaesthesia state

in folk medicine in sub-Saharan Africa and Middle East to treat skin and eye diseases, for example, Trichoma [16, 28]. Traditionally, desert truffles have been used as folk medicine in the Arabian countries over two millennia without any known complications [85]. In these countries, boiled truffle water extract has been recommended by the Bedouins for the treatment of trachoma, one of the earliest recorded eye diseases by the World Health Organization.

Spiritually, the Khoisan hunters in the Kalahari desert believe that desert truffles (*kuuste or n'xaba*) counteract the effects of poisoned arrows in shot animals [82], and until the animal is confirmed to be dead, the hunter can take no food or drink other than water. This is due to the fact that they believe that if the archer eats food, the wounded animal will regain its health and escape. Moreover, the hunters keep a piece of *kuuste* to eat as an antidote in case of being accidently wounded by a poisoned arrow.

7. Conclusions and Future Prospects

Africa and Middle East are very rich regions of unique types of macrofungi. Both truffles and mushrooms of this area of the world produce wide variety of interesting bioactive compounds of high medical value and were used for millennium in the treatment of different diseases. The main drawbacks for their application in modern medicine and for production in industrial scales are based on four main facts. First, most of these types of organisms are not cultivable in green house and thus their availability is seasonal and highly affected by climate change. The second fact is the wide variability of the bioactive ingredient contents which are highly dependent on collection time, procedure, season, and environment. Third, based on the chemical composition of both of mushrooms and truffles, they have high capacity to accumulate high concentration of heavy metals and radioactive isotopes. Thus, special consideration should be taken into account when collected from polluted areas. The fourth fact is the lack of standard testing protocols to guarantee the quality and the efficacy of the fungal product. Thus, more research is required to solve the above mentioned problem to increase the use of wild macrofungus in medical applications. This will change in part the current medical practice using chemically synthesized compounds of many side effects.

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Research Article

Antistress Effects of the Ethanolic Extract from *Cymbopogon schoenanthus* Growing Wild in Tunisia

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This study aimed to investigate the antistress properties of the ethanol extract of *Cymbopogon schoenanthus* (CSEE), growing wild in the southern part of Tunisia. The effect of extracts on H_2O_2-induced cytotoxicity and stress in human neuroblastoma SH-SY5Y cells. Its effect on stress-induced in ICR mice was exposed to force swim and tail suspension, in concordance with heat shock protein expression (HSP27 and HSP90), corticosterone, and catecholamine neurotransmitters level. Our results demonstrated that pretreatment of SH-SY5Y cells with CSEE at 1/2000, 1/1000, and 1/500 v/v dilutions significantly inversed H_2O_2-induced neurotoxicity. Moreover, CSEE treatments significantly reversed heat shock protein expression in heat-stressed HSP47-transformed cells (42°C, for 90 min) and mRNA expression of HSP27 and HSP90 in H_2O_2-treated SH-SY5Y. Daily oral administration of 100 mg/kg and 200 mg/kg CSEE was conducted to ICR mice for 2 weeks. It was resulted in a significant decrease of immobility time in forced swimming and tail suspension tests. The effect of CSEE on animal behavior was concordant with a significant regulation of blood serum corticosterone and cerebral cortex levels of catecholamine (dopamine, adrenaline, and noradrenaline). Therefore, this study was attempted to demonstrate the preventive potential of CSEE against stress disorders at *in vitro* and *in vivo* levels.

1. Introduction

Stress is known to induce alterations in various physiological responses even leading to pathological states [1]. It was demonstrated that different stress paradigms [2, 3] significantly affected learning and memory function and intensified fear memory in mice. The effects are supposed to be an outcome of a complex interaction of stress and altered activity of different mechanisms such as decrease in central neurotransmitters, neurohormonal factors [4], and neurotrophic factors [5] and increase in free radical generation and oxidative damage in the central nerve system [6].

In view of the potential use of natural products and botanicals as stress adaptogens, antioxidant rich phytochemical extracts are gaining a lot of interest. In this respect, several plants are traditionally and clinically used for the management of neurological disorders. From our previous research and others, several plants and herb preparations were demonstrated to protect against neuronal damage [7], Parkinson and Alzheimer' disease [8, 9], to improve neuronal differentiation [10], and to act against depression [11, 12], epilepsy [13], and anxiety [14].

Recent surveys reported that psychiatric conditions especially stress and depression were among the most common mood pathologies treated with complementary and alternative therapies [15, 16]. This correlates with a worldwide increasing trend to integrate traditional medicine with primary health care, because of its "green image", its cultural significance, and its accessibility to all societal categories [17].

Herbal medicines are an important part of the culture and traditions in the African continent where around 122 drugs

originating from 94 species have been discovered through ethnobotanical leads [18]. North Africa and Sahara are known by their richness in medicinal plants which are gathered and used as greens, spices, and condiments [19]. About 70% of the wild plants in North Africa are known to be of potential value in fields such as medicine and biotechnology.

Tunisia has a large plant biodiversity. Actually, over 350 species are considered herbal, medicinal, and aromatic plants (HMAP). These resources are used, mainly by rural communities for traditional phytotherapy, and practiced for several generations and civilizations [20]. Preservation of these species and knowledge of their uses require specific intervention, and these resources should not be lost due to environmental degradation, agricultural expansion, and urbanization. In this respect, this study was conducted to elucidate the traditional usage of *Cymbopogon schoenanthus* L. spring (CS).

CS is an aromatic culinary herb grown in the southern area of Tunisia, and it is locally known as "El bekherai". It is used for several preparations of meat and salad or served with tea because of its pleasant aroma appreciated by north African inhabitants [21]. Besides its use in culinary preparations, CS is also used in folk medicine. Its decoction and infusion are taken as diuretic to reduce intestine spasm and to act against food poisoning, antirheumatism, antianorexia, and digestive [22]. Our recent research demonstrated that CS extracts and essential oil have antioxidant and acetylcholinesterase inhibitory properties [23, 24].

Using *in vitro* bioassay and *in vivo* models, the antistress effect of ethanol extract of *Cymbopogon schoenanthus* (CSEE) was evaluated with a focus on the heat shock protein role in protecting neuronal cells against H_2O_2-induced cytotoxicity in SH-SY5Y cells, corticosterone, and catecholamine regulated levels in stressed-mice. Human neuroblastoma SH-SY5Y cells and HSP47-transformed cells were used as *in vitro* model; tail-suspended and forced-swim ICR mice were used as *in vivo* model.

2. Materials and Methods

2.1. Plant Extracts Preparation. *C. schoenanthus* was collected from the south of Tunisia in February 2011. The leaves were allowed to dry on the shadow at 25°C, and then they were ground into fine powder. 100 g of the powdered leaves was soaked in 1000 mL of ethanol (70%), for 2 weeks. The liquid fraction was centrifuged, filtered at 0.22 μm, and kept at −80°C.

2.2. HPLC Analysis of CSEE. Diluted samples were injected to RP-HPLC. The separation of phenolic compounds was performed with an Agilent 1100 series HPLC system equipped with online degasser (G 1322A), quaternary pump (G 1311A), a thermostatic autosampler (G 1313A), column heater (G1316A), and diode array detector (G 1315A). Instrument control and data analysis were carried out using Agilent HPLC Chemstation 10.1 Edition of Windows 2000. The separation was carried out on a reverse phase ODS C18 (250 mm × 4.6 mm ID, 5 μm particle size Hypersil) column used as stationary phase at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water sulphuric acid (0.2%) (Solvent B). The flow rate was kept at 0.5 mL·min^{-1}. The gradient program was as follows: 15% of A/85% of B at 0–12 min, 40% of A/60% of B at 12–14 min, 60% of A/40% of B at 14–18 min, 80% of A/20% of B at 18–20 min, 90% of A/10% of B at 20–24 min, and 100% of A at 24–28 min. The injection volume was 20 μL, and peaks were monitored at 280 nm. Peak identification was obtained by comparing the retention time and the UV spectra of sample phenolic chromatogram with those of pure standards from Sigma (St. Louis, MO, USA). Analyses were performed in triplicates.

2.3. Cell Culture. Human neuroblastoma SH-SY5Y cells were cultured in 1 : 1 mixture of Dulbecco's minimum essential medium (DMEM; Sigma, USA) and Ham's F-12 nutrient mixture (Sigma, USA) supplemented with 15% fetal bovine serum (FBS; Sigma, USA), 1% nonessential MEM amino acid, and 1% penicillin (5000 μg/mL)-streptomycin (5000 IU/mL) solution (ICN Biomedical, Inc.). The cells were cultured in 100 mm dishes and passaged by trypsinization using 0.25% Trypsin-EDTA (Sigma) at 80% confluence twice a week. Medium was changed every other day, and cells were incubated at 37°C (5% CO_2).

Chinese hamster ovary (CHO) cells, stably transfected with (+) or without (−) HSP47 promoter, were cultured in F12 medium supplemented with 10% FBS, 0.2% Kanamycin solution, and 0.1% G418 (Gibco BRL 13075-015). The cells were cultured in 75 cm^2 T-flasks and passaged by trypsinization at 80% confluence. Medium was changed every two days, and cells were incubated at 37°C (5% CO_2).

2.4. Neuroprotective Properties of CSEE against H_2O_2-Induced Cytotoxicity in SH-SY5Y Cells. SH-SY5Y cells were seeded at 1×10^4 cell/well in 96 well microplates and allowed to attach for 24 h incubation. Then cells were treated with CSEE (1/100, 1/1000, and 1/10000) for 72 h to investigate the noncytotoxic concentrations or in combination with H_2O_2. In H_2O_2 treatment panel, SH-SY5Y cells were pretreated with CSEE at 1/2000, 1/1000, and 1/500 for 24 h, followed by treatment with 150 μM H_2O_2 for 24 h. Cell viability was performed using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) as explained in our previous research [10]. Briefly, 10 μL of MTT solution (5 mg/mL in PBS (−)) was added to each well of 96-well plates and incubated for 6 h at 37°C in a 95% humidified air—5% CO_2 incubator. Then the formazan was formed and later dissolved in 10% sodium dodecyl sulfate (SDS). The absorbance was determined at 570 nm using blanks (wells which contain a mixture of extract and medium without cells) as a reference. Cell viability was reported as a percentage of control cells (cells treated with medium only).

2.5. HSP47 Assay. HSP47-transformed cells were plated at 1×10^4 cells/well in 100 μL of culture medium and allowed to attach and grow for 48 h at 37°C (5% CO_2). Then, the cells were subjected to a heat shock for 90 min at 42°C, and recovered at 37°C during 2 h. After the recovery time,

cells were treated with CSEE (1/1000, 1/500, and 1/100 v/v dilutions) and incubated for 3 h at 37°C, in 5% CO_2 incubator. Then the cells were washed twice with PBS (−), and HSP47 assay was performed as described in our previous study [25]. Moreover, the effect of CSEE on HSP47-transformed cell viability was investigated using MTT assay as previously described.

2.6. Effect of CSEE on HSP27 and HSP90 mRNA Expression in H_2O_2-Treated SH-SY5Y Cells. SH-SY5Y cells were plated at 2×10^5 cells/mL in 100 mm dish and allowed to attach for 24 h, then treated with various concentrations of CSEE at 1/500, 1/1000, and 1/2000 for 24 h, and then cotreated with 150 μM H_2O_2 for 24 h. Cells were washed with cold PBS, and total RNA was extracted using Isogen Kit (Wako, Japan). Total RNA was quantified by Thermo scientific Nano drop 2000 (USA). Reverse transcription reactions were performed using the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) [11].

All Primer sets: HSP27 (Hs03044127_g1), HSP90 alpha (Hs00743767_sH), and GAPDH and TaqMan probes for experimental genes were analyzed using Applied Biosystems. HSP27 and HSP90 mRNA expression was quantified using TaqMan real-time quantitative PCR (AB 7500 fast real-time system, Applied Biosystems, U.S.A.). Amplifications were performed in 20 μL final volume as explained in our previous study [11]. Gene expression was normalized to GAPDH and reported as fold of control.

2.7. Animal Treatment. Sixty-four ICR mice weighing between 26–30 g at 5 weeks of age (Charles River, Japan) were used for *in vivo* experiments. The animals were individually housed and allowed to acclimatize with free access to food and water for extra one week under a 12/12 light dark cycle, with controlled temperature (23°C) and humidity (40–60%). After acclimatization, animals were assigned to 2 groups of 32 animals each that were used respectively for TST and FST. Each group was randomized into 4 subgroups of 8 animals each: (1) vehicle control group (administered with distilled water), (2) imipramine group (administered with 15 mg/kg imipramine), (3) CSSE 100 group (administered with 100 mg/kg CSSE), and (4) CSSE 200 group (administered with 200 mg/kg CSSE).

Imipramine and *C. schoenanthus* dried ethanol extract were freshly dissolved in distilled water and were orally administered (p.o) to mice in a volume of 10 mL/kg body weight daily for 13 consecutive days. One hour prior to extract and drug administration, all mice were deprived from food but not water; in other time periods, all animals had free access to food and water [26].

2.8. Tail Suspension Test. The tail suspension test (TST) was based on the method of Steru et al. [27] with minor modifications. Briefly, all mice were individually suspended by the tail with clamp (1 cm distant from the top) for 6 min in a box (35 × 70 × 50 cm) with the head 5 cm to the bottom. Testing was carried out in a darkened room with minimal background noise, 1 h after plant extract and 30 min after imipramine administration. The duration of immobility time was considered during the final 3 min interval.

2.9. Forced Swimming Test. The forced swimming test (FST) was conducted as previously described by Porsolt et al. [28]. Briefly, each mouse was placed in a 25 cm glass cylinder (14 cm diameter) filled with water up to 15 cm, maintained at 24 ± 2°C, and forced to swim for 6 min (before the swimming session). Then mice were removed and dried. They were again forced to swim in a similar environment for a period of 6 min and 24 h later (test session). Immobility duration was recorded using a camera during the last 3 min of the 6 min tests. Extracts were administered 1 h before the forced swimming test, or imipramine was administered 30 min before the test. In the subchronic treatment study, the same dosages of extracts or imipramine, as those in the acute treatment study, were administered once a day for 2 weeks, and the final treatment was conducted 1 h (extracts) or 30 min (imipramine) before behavioral tests.

2.10. Determination of Corticosterone in CSEE-Treated ICR Mice Blood Serum. Serum corticosterone (CORT) levels in the control and CSEE-treated mice were determined using an enzyme immunoassay kit (AssayMax Corticosterone ELISA Kit, AssayPro LLC) according to manufacturer's recommendations. Blood serum was collected by centrifugation at 3000 xg for 10 min, then it was diluted at 1/200 with EIA diluent and immediately stored at −20°C until use. 25 μL of different serum samples or standard solution was mixed with 25 μL of biotinylated corticosterone in each well and allowed to stand for 2 h at room temperature. Then wells were washed and incubated with 50 μL of streptavidin-peroxidase conjugate for 30 min; afterwards, wells were washed again and incubated with 50 μL of chromogen substrate solution for 30 min until the optimal blue color density develops. Then, 50 μL of stop solution was added to each well, and finally, the absorbance in each well was recorded at 450 nm. The level of CORT was also calculated using standard curve and reported in ng/mL.

2.11. Determination of Dopamine (DOP), Adrenaline (ADR), and Noradrenaline (NAD) in CSSE-Treated ICR Mice Brain Tissue. Cerebral cortex catecholamine (Dopamine (DOP), adrenaline (ADR), and noradrenaline (NAD)) levels were determined using 3-CAT Research ELISA (Labor Diagnostika Nord GmbH & Co. KG) according to manufacturer's instructions. Briefly, 100 mg of brain tissue was homogenised. Also 60 μL of brain sample or standards were mixed with 25 μL of TE buffer into all wells, and the plate was shaken for 1 h at room temperature. After washing twice, 150 μL of acylation buffer and 25 μL of acylation reagent were added to each well. Then wells were shaken, washed, and incubated with 200 μL of hydrochloric acid for 10 min at room temperature. 190 μL from each well were taken and incubated for 2 h at 37°C with 50 μL of enzyme solution. Afterwards, 75 μL of samples from enzyme plate were mixed with 50 μL of the respective antiserum into all wells and incubated for 18 h at 4°C. Plates were incubated for 30 min

at room temperature after washing and adding 100 μL of enzyme conjugate in each well. Then, wells incubated with 100 μL of substrate for 30 min at room temperature and 50 μL of stop solution was added. Finally, the absorbance in each well was recorded at 450 nm and the level of catecholamine was calculated using standard curve and reported in ng/mL.

2.12. Statistical Analysis. All data were expressed as mean ± S.E.M, and significances were calculated using Student's t-test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. HPLC Analysis. Seven phenolic compounds were successfully identified in CSEE based on the retention time and spectral characteristics of their peaks against those of the standards using RP-HPLC coupled with UV-Vis multiwave length detector. The chromatogram of plant extract was compared to authentic standards of phenolic acid and flavonoid profiles, which allowed us to identify 5 phenolic compounds at 330 nm: quercetin-3-rhamnoside, trans-cinnamic acid, resorcinol, caffeic acid, and 2.5-dihydroxybenzoic acid and 2 others phenolic compounds at 280 nm: ferulic acid and gallic acid (Table 1).

3.2. CSEE Protects SH-SY5Y Cells against H_2O_2-Induced Toxicity. To determine the noncytotoxic concentrations of CSEE, SH-SY5Y cells were treated with CSEE at 1/10000, 1/1000, and 1/100 v/v dilution for 72 h. CSEE did not affect SH-SY5Y cell viability. However at 1/100 dilution cell viability slightly decreased, but this decrease was not significant (Figure 1(a)).

In preliminary experiments, 150 μM H_2O_2 was found to be the challenging concentration to reduce SH-SY5Y cells by 40% after 24 h exposure (data not shown). Pretreatment of SH-SY5Y cells with CSEE (1/2000, 1/1000, and 1/500 v/v dilutions) significantly and dose dependently improved cell viability in H_2O_2-treated cells to reach 77 ± 8.53%, 91 ± 6.66%, and 90 ± 5.19% of control, respectively.

3.3. CSEE Reduce Heat Stress Effect in HSP47 Transformed Cells. To investigate the antistress effect of CSEE, HSP47-transformed cells were used as model. Cells were heat-shocked for 90 min at 42°C, 5%CO_2 and recovered for 2 h at 37°C, 5%CO_2. Then cells were exposed to CSEE at 1/1000, 1/500, and 1/100 v/v dilutions for 3 h. CSEE significantly and dose dependently reduced HSP47 expression by 81.4 ± 4.64%, 55.36 ± 9.28%, and 40.7 ± 5.11% of control, respectively without cytotoxic effect (Figure 2).

3.4. CSEE Pretreatment Reduces the H_2O_2-Stimulated mRNA Expression of HSP27 and HSP90 in SH-SY5Y Cells. To assess the mechanism by which CSEE reversed H_2O_2-induced toxicity, we investigated the effects of CSEE pretreatments on heat shock proteins HSP27 and HSP90 mRNA expression in H_2O_2-treated SH-SY5Y cells. H_2O_2 treatment increased HSP27 and HSP90 by 5 ± 0.40 and 3.5 ± 0.38 fold of control, respectively. When pretreated with CSEE (1/2000, 1/1000, and 1/500) for 24 h, HSP27 mRNA expression significantly decreased to reach the control level. HSP90 mRNA significantly decreased only in 1/1000 and 1/500 dilution treatments to reach 2 ± 0.21 fold of control (Figure 3).

3.5. CSEE Oral Administration Reduced Immobility Time in the TST and in the FST. CSEE at 100 and 200 mg/kg (p.o) caused neither the death of any animal nor the change in mice coat color. Moreover, CSEE doses (p.o) did not reduce body weight in TST or FST significantly (data not shown).

The antistress effect of orally administered CSEE in ICR mice was investigated for 13 consecutive days by recording immobility time in TST and FST.

The baseline of immobility time (day 0, prior to CSEE (p.o)), shows 2 clusters (Figure 4). However, on day 1, the immobility time did not show any significant difference among the four animal groups. The TST was assessed every four days, and starting from day 5 the immobility time was increased in control and imipramine groups to reach 81 ± 2.7 sec and 75 ± 3.0 sec on day 13. In CSEE-treated groups, 100 mg/kg and 200 mg/kg doses significantly maintained the immobility time in ICR mice shorter than that in vehicle and imipramine groups during all the period of the experiment. In fact, both 100 and 200 mg/kg CSEE resulted in 48 ± 2.5 sec on day 13 with a similar trend. The effects of CSEE appeared to be more potent than those of imipramine during the last days of treatment.

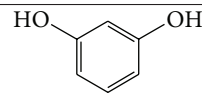
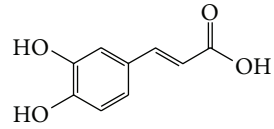
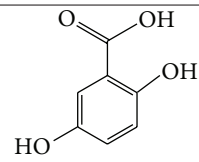
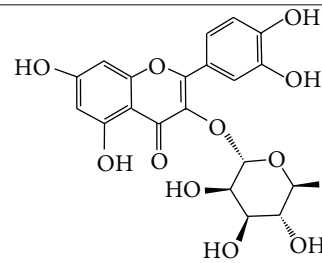
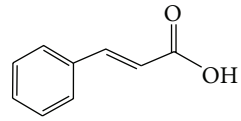
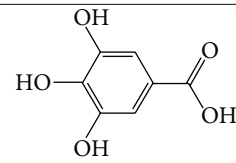
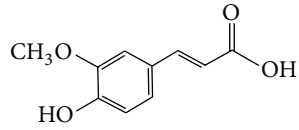
Similarly to TST, FST was assessed every four days during 2 weeks. The immobility time was increased in the control group to reach 82 ± 4 sec in the last day of treatment. In imipramine group, the immobility time was decreased to reach 53 ± 1.6 sec on day 5, then it was increased to reach 75 ± 2.4 sec on the day 13 (Figure 5). However, CSEE at 100 and 200 mg/kg significantly reduced the immobility time to reach 45 ± 0.8 sec on D13.

3.6. Effects of CSEE on Serum Corticosterone Levels. The swim stress procedure evoked a significant increase in serum CORT levels 373 ± 0.3 ng/mL in the control group (Figure 6). CSEE, at 100 mg/kg and 200 mg/kg, and imipramine (15 mg/kg) significantly reduced the serum CORT levels in FST-induced stress in mice to 92 ± 1.3, 91 ± 3.5, and 69 ± 2.7 ng/mL, respectively.

Similarly with FST, a significant increase in serum CORT concentrations was observed in control mice exposed to TST. Further, 100 and 200 mg/kg of CSEE and imipramine (15 mg/kg) significantly reduced the serum CORT levels in mice to 160 ± 2.7, 42 ± 2.5, and 116 ± 2.5 ng/mL, respectively (Figure 6).

3.7. Effect of CSEE on Monoamine Neurotransmitters Levels in Brain. DOP, ADR, and NAD were significantly decreased to 5.1 ± 0.07, 0.3 ± 0.05, and 25.2 ± 0.04 ng/mL, respectively, in stressed mice when exposed to tail suspension test (Table 2). CSEE at 100 mg/kg and 200 mg/kg and imipramine (15 mg/kg) significantly increased the DOP, ADR, and NAD levels (Table 2). A significant increase of monoamine neurotransmitter levels was observed in treated groups' brain in FST. However, in control group, DOP, ADR, and NAD

Table 1: Major compounds of *C. schoenanthus* identified by HPLC.

RT	Compounds	Structure	Percentage (%)	Detection wave length (nm)
9,674	Resorcinol		5.08	330
10,103	Caffeic acid		3.18	330
11,708	2.5-Dihydroxybenzoic acid		2.01	330
13,463	Quercetin-3-rhamnoside		32.56	330
19,124	Trans-cinnamic acid		9.72	330
5,58	Gallic acid		8.80	280
13,463	Quercetin-3-rhamnoside		32.82	280
13,931	Ferulic acid		9.83	280

were significantly decreased to 6.1 ± 0.02, 0.2 ± 0.07, and 45.6 ± 0.03 ng/mL respectively.

4. Discussion

The present study investigated the potential preventive effect of CSEE against stress in H_2O_2-treated SH-SY5Y cells, in heat-stressed HSP47-transformed cells, and in two mouse models of despair tasks: the TST and the FST. Moreover, neurobiochemical and neuropharmacological paradigms were used as tools to contribute to the understanding of the involvement of heat shock proteins (HSP27 and HSP90), catecholamines neurotransmitters (dopamine, adrenaline, and noradrenaline), and corticosterone in the antistress-like activity of CSEE.

It is well known that chronic exposure to stress is causative factor of free radical generation and reactive oxygen species elevation in the body [6]. Oxidative stress is well demonstrated to be a leading factor in neuronal cell death and damage [29]. Thus, antioxidants intake might be an effective strategy to protect the human body against stress-mediated pathologies. In this respect, phytochemicals, botanicals, and plant extracts are gaining a lot of interest as complementary supplements to fight against oxidative stress. In the current study, CSEE, a polyphenol-rich plant extract, treatment showed a significant protective effect against H_2O_2-induced

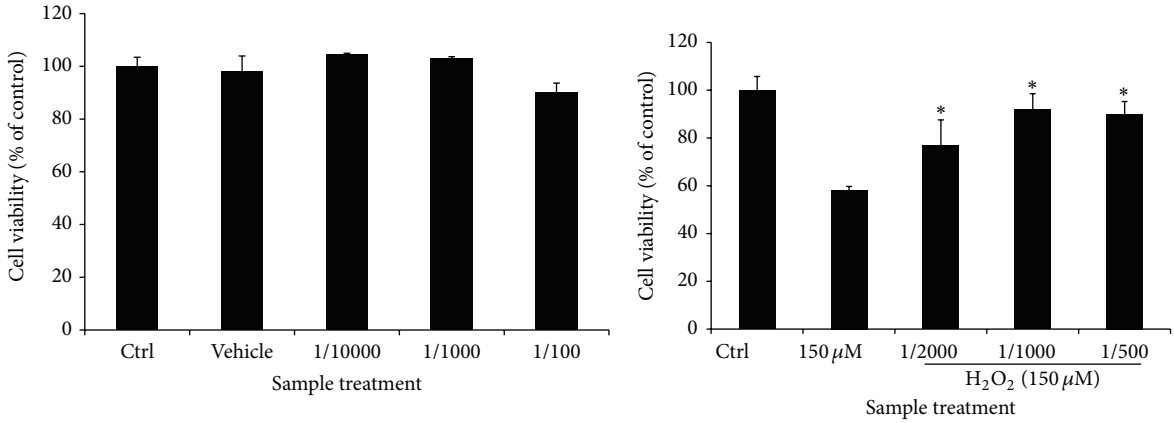


Figure 1: *Neuroprotective properties of CSEE against H_2O_2-induced cytotoxicity*. SH-SY5Y cells were seeded at 1×10^4 cell/well in 96-well microplate and treated with (a) CSEE (1/10000, 1/1000, and 1/100 v/v dilutions) for 72 h, (b) SH-SY5Y cells were pretreated with CSEE (1/10000, 1/1000 and 1/500 v/v dilutions) for 24 h and then treated with 150 μM H_2O_2 for 24 h. Cell viability was determined using MTT assay as explained in Materials and Methods. Each bar represents the mean of 3 independent trials ± SD. $^*P < 0.05$, $^{**}P < 0.01$ versus control cells, (Student's *t*-test).

Table 2: *Effect of CSEE on DOP, ADR, and NAD levels (ng/mL) in the brain of ICR mice*. Mice fed with 100 and 200 mg/kg of CSEE for 14 days were sacrificed by dislocation of the cervical spine one day after the last behavioral test, and then the brain samples were rapidly washed with PBS (−) and stored at −80°C until use. Monamine neurotransmitters levels were quantified using ELISA kit as explained in Section 2.

Groups	TST			FST		
	DOP	ADR	NAD	DOP	ADR	NAD
Control	5.138 ± 0.07	0.365 ± 0.06	25.196 ± 0.05	6.147 ± 0.02	0.238 ± 0.07	45.655 ± 0.03
Imipramine	6.395 ± 0.08**	0.59 ± 0.03*	51.247 ± 0.04**	6.713 ± 0.06**	0.586 ± 0.02**	50.822 ± 0.05
100 mg/kg	6.378 ± 0.08**	0.551 ± 0.03*	48.640 ± 0.07**	6.717 ± 0.02**	0.584 ± 0.08**	52.167 ± 0.04
200 mg/kg	6.427 ± 0.02**	0.504 ± 0.02*	53.696 ± 0.03**	6.655 ± 0.06**	0.599 ± 0.02**	49.020 ± 0.06

With, DOP: dopamine, ADR: adrenaline, and NAD: noradrenaline.
Each value represents the mean ± SD ($n = 4$). $^*P < 0.05$, $^{**}P < 0.01$ versus Control group (Student's *t*-test).

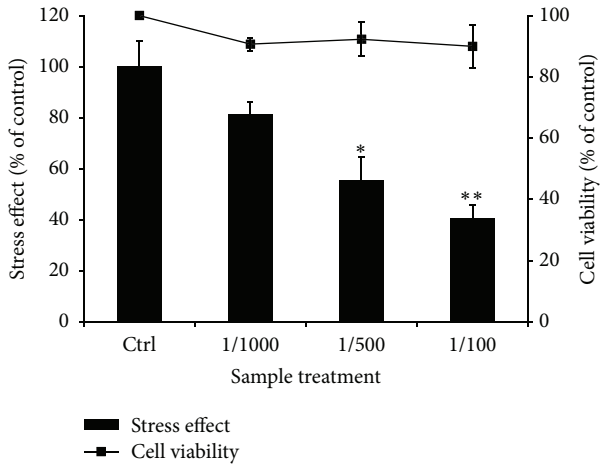


Figure 2: *Effect of CSEE on HSP47 expression and cell viability in Chinese hamster ovary transformed cells*. Cells were seeded at 1×10^4 cell/well in 96-well microplate and treated with CSEE at 1/1000, 1/500, 1/100 v/v dilution. Cell viability and HSP47 expression were performed as explained in Materials and Methods. Each bar represents the mean of 3 independent trials ± SD. $^*P < 0.05$, $^{**}P < 0.01$ versus control cells, (Student's *t*-test).

toxicity in SH-SY5Y cells and against heat stress-induced in HSP47-transformed cells. Moreover, CSEE significantly reversed HSP27 and HSP90 mRNA expressions in H_2O_2-treated SH-SY5Y cells.

A probable underlying mechanism of this protection may be associated with the presence of flavonoids (Resorcinol and Quercetin-3-rhamnoside) and phenolic acids (Trans-cinnamic acid, Ferulic acid and Gallic acid). In our study, we demonstrated that CSEE protection against H_2O_2-induced stress was regulated by heat shock proteins (HSP). In fact, HSPs provide a fundamental mechanism to defend neuronal cells against the effect of diverse stressors like temperature, oxidation, inflammation, xenobiotics, irradiation, and pollutants [25]. HSPs are becoming a therapeutic target in neurodegenerative diseases and aging because the pathogenesis mechanism of these diseases is thought to be related to an abnormal increase of unfolded protein response [30]. Beside its role against protein aggregation, HSP27 is reported to have the capacity to sequester cytochrome C when released from the mitochondria into the cytosol and to have important antioxidant properties [31] by decreasing the abundance of reactive oxygen species (ROS) [32] and

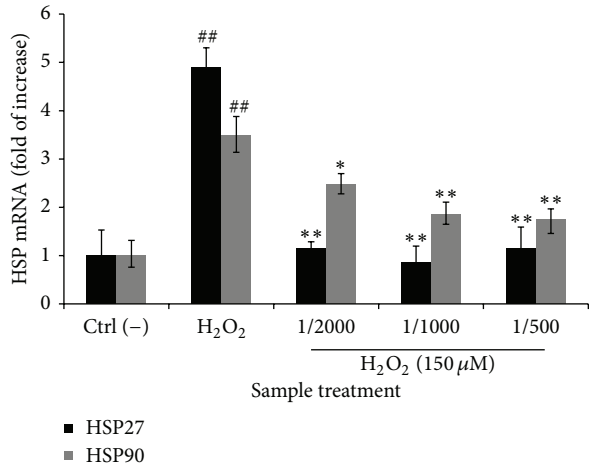


Figure 3: *Effect of CSEE on mRNA expression of HSP27 and HSP90 in H_2O_2-treated SH-SY5Y cells*. SH-SY5Y cells were seeded at 2×10^5 cell/mL in 100 mm dish and was treated with CSEE (1/500, 1/1000 and 1/2000) for 24 h, and then they were treated with 150 μM H_2O_2 for 24 h. The mRNA expression of genes was normalized to GPDH mRNA expression and expressed as ratio of control. Each bar represents the mean of duplicate ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ versus positive control group, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control group (Student's *t*-test).

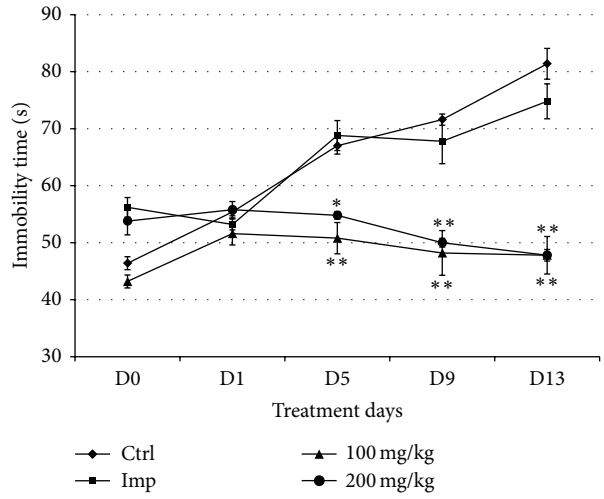


Figure 4: *Effect of administration of CSEE on the immobility time in the TST*. ICR mice were orally administrated with distilled water in control group, 15 mg/kg imipramine, and 100 and 200 mg/kg CSEE. The immobility time in the TST was calculated as reported in Materials and Methods. Data are represented as the average of 5 observations ± SEM. $^{*}P < 0.05$ versus Control group (Student's *t*-test).

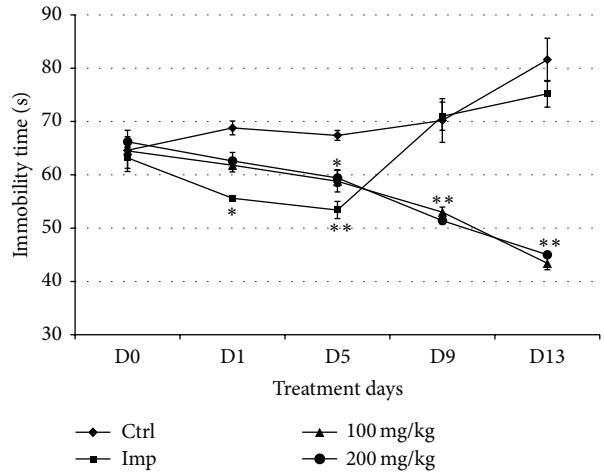


Figure 5: *Effect of administration of CSEE on the immobility time in the FST*. ICR mice were orally administered with distilled water in control group, 15 mg/kg imipramine in positive control group, and 100 and 200 mg/kg of CSEE. The immobility time in the FST was calculated as reported in Materials and Methods. Data are represented as the average of 5 observations ± SEM. $^{*}P < 0.05$ versus control group (Student's *t*-test).

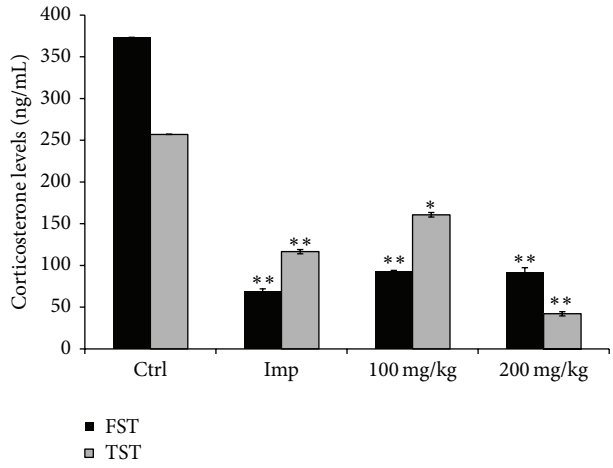


Figure 6: *Effect of CSEE on serum level of corticosterone in ICR mice subjected to FST and TST*. Mice fed with 100 and 200 mg/kg of CSEE for 14 days were sacrificed by spinal cord dislocation one day after the last behavior test; then the blood sample was collected, and the serum was rapidly separated. Plasma corticosterone was measured using Assay Max Corticosterone ELISA kit as explained in Materials and Methods. Each value represents the mean of ±SD ($n = 4$). $^{*}P < 0.05$ versus control group (Student's *t*-test).

maintaining glutathione in its reduced form [33]. Moreover, HSP27 is involved in the cell survival mechanism since its depletion was demonstrated by Rocchi et al. [34] to result in cell apoptosis through caspase3 activation. On the other hand, it was reported that HSP90 acts on misfolded proteins induced by conditions such as heat and the presence of ROS. In addition, in the absence of HSP90, many clients are turned over when misfolded or misassembled [35]. Same authors suggested that "stress" moves cells away from homeostasis and leads to an increase of the load for HSP90 and its co-chaperones.

Since the first emergence of "stress", researchers tried to find and develop several animal models to solve this problem. The tail suspension and forced swimming tests are the most common predictive tests for screening the beneficial properties of medicinal plants [27, 28].

In traditional medicine, *C. schoenanthus* was used for several treatments. However, its antistress effect is still not yet established *in vivo*. Within the same genus of *Cymbopogon, C. citratus* essential oil was reported to have an anxiolytic activity [36, 37]. Moreover, Quintans-Júnior et al.

[38] demonstrated the anticonvulsant effect of the essential oil from *C. winterianus*. The present study was the first to demonstrate that oral administration of *C. schoenanthus* ethanol extract (100 and 200 mg/kg, p.o.) significantly reduced immobility time in the TST and FST in mice compared to vehicle group and the classical tricyclic antidepressant, imipramine (15 mg/kg, p.o.) treated group. The immobility time in TST and FST, referred to behavioral despair in animals, is believed to reproduce a condition similar to human depression [39]. Thus, a reduction in the total duration of immobility indicates an antistress effect [27, 40]. Moreover, the effect of CSEE on animal behavior was concordant with its effect on blood corticosterone and cerebral cortex monoamine levels. In fact, CSEE oral administration at 200 mg/kg significantly decreased corticosterone levels to 42 ± 2.5 ng/mL and improved DOP, ADR, and NAD to 6.6±, 0.6±, and 53.7± ng/mL, respectively. In this respect, from our and others research, there is a great interest in phytochemicals and dietary molecules that may interact with the hypothalamic-pituitary-adrenal (HPA) axis and the monoamenergic, catecholaminergic, and cholinergic systems [11, 41]. The precise mechanisms by which CSEE produced its antistress effects were not fully understood. However, according to our results, this effect could be regulated by its interaction with the monoaminergic system and HPA axis. In this context, it is already suggested that the first step of antistress effect mechanism should be considered as an increase in the monoamine levels at the synapse [42] and a decrease in corticosterone serum levels [43].

Taken together, our results demonstrated that CSEE was effective in producing significant antistress effects at *in vitro* and *in vivo* levels. The molecular mechanism by which CSEE exhorted its beneficial effects seems to be partially modulated by chaperone activation, monoaminergic system, and HPA axis regulation. Furthermore, the *in vivo* results of CSEE oral administration showed a comparable effect to the established commercial antidepressant drug (imipramine). The decrease of immobility time was dose dependent in two models. These results indicated that CSEE had a dose-dependent antistress effect that was comparable to established commercial antidepressant drugs.

The HPLC analysis of CSEE identified several compounds with known neuroprotective activities like caffeic acid, ferulic acid, and quercetin. However, future studies should be addressed in exploring their mixture and the molecular mechanism that may regulate their activities.

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Research Article

In Vitro Antiproliferative Effect of *Arthrocnemum indicum* Extracts on Caco-2 Cancer Cells through Cell Cycle Control and Related Phenol LC-TOF-MS Identification

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This study aimed to determinate phenolic contents and antioxidant activities of the halophyte *Arthrocnemum indicum* shoot extracts. Moreover, the anticancer effect of this plant on human colon cancer cells and the likely underlying mechanisms were also investigated, and the major phenols were identified by LC-ESI-TOF-MS. Results showed that shoot extracts had an antiproliferative effect of about 55% as compared to the control and were characterised by substantial total polyphenol content (19 mg GAE/g DW) and high antioxidant activity (IC_{50} = 40 μg/mL for DPPH test). DAPI staining revealed that these extracts decrease DNA synthesis and reduce the proliferation of Caco-2 cells which were stopped at the G_2/M phase. The changes in the cell-cycle-associated proteins (cyclin B1, p38, Erk1/2, Chk1, and Chk2) correlate with the changes in cell cycle distribution. Eight phenolic compounds were also identified. In conclusion, *A. indicum* showed interesting antioxidant capacities associated with a significant antiproliferative effect explained by a cell cycle blocking at the G_2/M phase. Taken together, these data suggest that *A. indicum* could be a promising candidate species as a source of anticancer molecules.

1. Introduction

Cell cycle progress is partially controlled by the balance between the accumulation of reactive oxygen species (ROS) and the antioxidant system [1]. A disturbance during the cell division can lead to abnormal cell proliferation. The overproduction of ROS results in oxidative stress, a deleterious process that can be an important mediator of cell structure damages and initiation of serious diseases such as cancer [2]. ROS are implicated in ischemia-induced permeability changes of the intestine, in Crohn's disease, and in ulcerative colitis [3]. Several approaches including apoptosis [4], autophagy [5], and differentiation [6] are used to control and eradicate cancer cells. Recently, the analysis of cell cycle arrest emerged as a novel approach for cancer eradication [7]. Cells recognise and respond to extracellular stimuli by engaging specific intracellular programs, such as the signalling cascade that leads to the activation of mitogen-activated protein kinases (MAPKs) [8]. All eukaryotic cells possess multiple MAPK pathways which coordinately regulate diverse cellular activities like gene expression, mitosis, metabolism, survival, and cell death [9]. The importance of MAPK pathways into cell proliferation and death is highlighted by the observation that deregulation of these kinase cascades can result in cell transformation and cancer [10].

Recently, three groups of mitogen-activated protein kinases (MAPKs) responsible for the extracellular stimuli response cascade were characterised in cells as extracellular

signal-regulated kinases (ErKs 1 to 5), c-Jun amino-terminal kinases (JNKs 1, 2, and 3), and p38 isoforms [11, 12]. MAPKs phosphorylate specific serines and threonines of target protein substrates and regulate cellular activities like gene expression, mitosis, movement, metabolism, and programmed death [12]. During cell division, checkpoint controls are characterised by a number of Cdk/cyclin families, which are responsible for the cell cycle progression [13]. Moreover, checkpoint kinases such as Chk1 and Chk2 are responsible for the mechanism involved in the induced cell cycle arrest [14]. The p38 MAPK pathway is implicated in the suppression of tumorigenesis, since it can (1) inhibit the cell growth by decreasing the expression of cyclin D [15], (2) inhibit the activity of Cdc25 phosphatases [16], and (3) engage the p16/Rb and $p19^{ARF}$/p53 tumour suppressor pathways [17]. Consequently, the p38 MAPK pathway is activated upon cellular stress and often engages process that can block proliferation (cell cycle arrest) or promote apoptosis. The extracellular signal-regulated kinases (ErKs) with the p38 pathways are all new molecular targets for therapeutic research [12]. MAP kinases inhibitors will certainly be the next developed mediators for the treatment of human disease [12].

The therapeutic effects of plants have been partly ascribed to their high content in bioactive molecules, such as phenolic compounds. To overcome oxidative stress generated by severe habitat conditions, plants produce these metabolites. This is the case of several halophyte species, which are used in folklore medicine, since the extracts proved to have activity against human, animal, and plant pathogens [18]. Phenols are an integral part of human diet due to their abundance in fruits and vegetables and have attracted considerable interest as powerful antioxidant compounds [19]. Besides, these compounds present large biological properties such as antimicrobial, antiviral, anti-inflammatory, antiallergic, antithrombotic, cardioprotective and vasodilatory effects [20]. For example, flavonoids, flavonolignans, isothiocyanates, and proanthocyanidins are known to play an important role in the cancer cell behaviour [18]. The protective effect of polyphenols from *Glycyrrhiza glabra* extracts against oxidative stress in human cancer cells was also documented [21].

Arthrocnemum indicum is a traditional medicinal halophyte common as salt marshes in Tunisia [18]. It is used in the treatment of poisonous snakebites and scorpion stings. It also plays a prominent role in traditional oriental medicine and ancient Indian medicine (Ayurveda). Here, we assessed mainly the effect of *A. indicum* shoot extracts on the human colon cancer Caco-2 cell proliferation through the control of the cell cycle. Caco-2 cell lines are frequently used as a model in order to study the anticancer effect [22], the inhibition of inflammatory mediators [23], and the degree of transepithelial resistance [24]. The present study aims at evaluating the effect of shoot extracts of the halophyte *A. indicum* on Caco-2 cell growth. The possible underlying mechanisms involving Erk1/2 and p38 MAP kinases on cell cycle arrest were investigated, and the major phenolics in shoot extracts were identified by LC-ESI-TOF-MS.

2. Materials and Methods

2.1. Sampling and Sample Preparation. *A. indicum* (Chenopodiaceae) shoots were harvested at full flowering stage from the sebkha of El Kelbia locality (20 km northeast Kairouan; superior semiarid bioclimatic stage; mean annual rainfall: 400 mm) in May 2010. The harvested shoots were rinsed with distilled water, left at room temperature for 7 days in the dark, and ground to fine powder. For the anticancer effect analysis, extracts were obtained by soxhlet extraction at a ratio of 20 g dry powder in 200 mL of 80% methanol. They were kept for 24 h at 4°C, filtered through a Whatman n°4 filter paper, evaporated under vacuum, and were stored at 4°C until analysis. For the anticancer effect analysis, 10 g of powder was added to 100 mL 80% methanol, stored for one week at room temperature in the dark, and then filtered through a Millipore filter (0.2 μm). After drying under vacuum, the powder was dissolved in DMSO to get 2.5% (2.5 mg powder in 100 μL DMSO) as stock concentration. Finally, extracts were stored at −80°C until analysis.

2.2. Quantification of Phenolic Fractions

(i) Total polyphenols: Folin-Ciocalteu reagent was used to determine the amount of total phenolics in methanolic extracts [19]. An aliquot of 125 μL of diluted extract was added to 500 μL of distilled water and 125 μL of the Folin-Ciocalteu reagent. The mixture was shaken before adding 1250 μL of Na_2CO_3 (7%) and adjusted with distilled water to a final volume of 3 mL. After incubation for 90 min at 23°C in the dark, the absorbance versus prepared blank was read at 760 nm. Total phenolic content was expressed as mg gallic acid equivalent per gram of dry weight (GAE/g DW) using a calibration curve with gallic acid, ranging from 0 to 500 μg/mL. All samples were analyzed in triplicate.

(ii) Total flavonoids: the measurement of flavonoid content in *A. indicum* shoots was based on the method described by Ksouri et al. [19]. An aliquot of the samples or (+)-catechin standard was added to test tubes containing 75 μL of a 5% $NaNO_2$ solution and mixed for 6 min. Then, 150 μL of 10% $AlCl_3$ solution was added. After 5 min at room temperature, 500 μL of 1 N NaOH was added. The final volume was adjusted to 2.5 mL with distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm against the blank where the sample was omitted. Total flavonoid content was expressed as mg catechin equivalent per gram of dry weight (mg CE/g DW), through the calibration curve of (+)-catechin, ranging from 0 to 500 μg/mL. All samples were analyzed in triplicate.

(iii) Total condensed tannins: the content of condensed tannin was determined according to Ksouri et al. [19]. Briefly, 50 μL of diluted shoot extracts was mixed with 3 mL of 4% vanillin solution in methanol and 1.5 mL hydrochloric acid (1 N). The mixture was

allowed to stand for 15 min, and the absorbance was measured at 500 nm against 80% methanol. Results were expressed as mg catechin equivalent per gram of dry weight (mg CE/g DW). All samples were analyzed in triplicate.

2.3. Determination of Antioxidant Assays

(i) Evaluation of total antioxidant capacity: an aliquot (100 μL) of plant extract was added to 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were then incubated at 95°C for 90 min. After the mixture has been cooled at room temperature, the absorbance was measured at 695 nm (Anthelie Advanced 2, SECOMAN) against a blank. The total antioxidant activity was expressed as mg GAE/g DW [19]. The calibration curve was established between 0 and 500 μg/mL. All samples were analyzed in triplicate.

(ii) Stable free radical scavenging capacity: DPPH (1,1-diphenyl-2-picrylhydrazyl) quenching ability of plant extracts was measured according to Ksouri et al. [19]. One milliliter of the extract at different concentrations was added to 250 μL of a 2 mmol/L DPPH methanol solution. The mixture was shaken vigorously and then left at room temperature for 30 min in the dark. The absorbance of the resulting solution was then measured at 517 nm and corresponded to the ability of extracts to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. The extract concentration required to cause a 50% inhibition is expressed as IC_{50} (μg/mL) and calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] * 100, \quad (1)$$

where A_0 is the absorbance of the control at 30 min and A_1 is the absorbance of the sample at 30 min. All samples were analyzed in triplicate

(iii) Iron reducing power: the iron (III) reductive capacity of the extract was assessed as described by Ksouri et al. [19]. Briefly, 1 mL of methanol extract was mixed with 2.5 mL phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL $K_3Fe(CN)_6$ solution (1 g/100 mL). After 20 min at 50°C, 2.5 mL trichloroacetic acid (10 g/100 mL) was then added, and the mixture was centrifuged for 10 min at 650 ×g. Finally, the upper layer fraction (2.5 mL) was mixed with 2.5 mL of ultrapure water and 0.5 mL of ferric chloride (0.1 g/100 mL). Absorbance was measured at 700 nm. Ascorbic acid was used as a positive control. The higher absorbance indicates a higher reducing power. EC_{50} value (μg/mL) is the effective concentration giving an absorbance of 0.5 for reducing power and was obtained from linear regression analysis. All samples were analyzed in triplicate.

2.4. Cell Maintenance. The human carcinoma Caco-2 cell line was isolated from the colon cancer of a 72-year old Caucasian male. Culture was maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Sigma), 1% nonessential amino acids (Cosmo Bio Co., LDT), and 1% penicillin (5000 IU/mL)-streptomycin (5000 μL/mL) solution (ICN Biomedicals) at 37°C under 5% CO_2 atmosphere.

2.5. Antiproliferative Effect by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. To investigate the antiproliferative effect, Caco-2 cells were seeded in 96-well plates at a concentration of 2×10^4 cells/mL in Dulbecco's modified Eagle's medium (DMEM). Cells were kept at 37°C under 5% CO_2 and treated with different concentrations of DMSO. *A. indicum* shoot extracts ranged between 0.01 and 100 μg/mL. After 72 h of treatment, 10 μL MTT solution (5 mg/mL) was added to the culture medium. After 24 h of incubation, the formazan produced was dissolved using 100 μL of 10% SDS solution (Wako). Absorbance was measured at 570 nm on a multidetection microplate reader [6]. Results shown represent the mean of three independent experiments.

2.6. DAPI Staining. During the analysis by fluorescence microscopy, cells (2×10^4 cells/mL) were incubated for 72 h with 100 μg/mL *A. indicum* shoots. Control cells were treated with 0.4% DMSO. Then, samples were washed 2 times with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. After washing two times with PBS and staining with DAPI (4,6-diamidino-2-phenylindole) solution, the chromosomes were analyzed [25]. Results represent the mean of three independent experiments.

2.7. Cell Cycle Analysis. The cell cycle analysis was assessed using guava flow cytometry (Guava Technologies). To determine the plant effect on the cancerous cell division, Caco-2 cells (2×10^4 cells/mL) were pretreated for 72 h with 100 μg/mL *A. indicum* extracts. Then, cells were washed with PBS, fixed with 70% ice-cold ethanol, and stored at −20°C until analysis. After removing ethanol, the cells were suspended in 500 μL of cell cycle reagent (Guava Technologies) and incubated in the dark at room temperature for 30 min [6]. The results represent the mean of three independent experiments.

2.8. Western Blotting. To evaluate the effects of *A. indicum* extracts on the expression of checkpoint protein kinases, 2×10^4 cells/mL were seeded for 72 h in culture dish with 100 μg/mL of *A. indicum* shoot extracts. The treated cells were washed with PBS and lysed by RIPA buffer (Sigma Aldrich Co.) with protease inhibitor cocktail (Sigma Aldrich Co.). The mixture was centrifuged at 12,000 ×g for 20 min at 4°C. The protein-containing supernatant was kept, and the quantification of the proteins was performed using

the Plus One 2D Quant kit (GE Healthcare). Proteins (20 μg) were resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane using the iBlot dry blotting system (Invitrogen). After blocking with 5% nondry fat milk, the membrane was incubated at 4°C overnight under shaking with the appropriate antibodies. The bands of cyclin B1, Erk1/2, pErk1/2, p38, pp38, β-actin, Chk1, pChk1, Chk2, and pChk2 proteins were detected by horseradish peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system ECL (Amersham Biosciences). After band staining, the gels were scanned and converted to images and then analyzed with ImageJ software (GE Healthcare). The images were rectified and transformed into binary images to calculate the relative density estimated as a percentage of band appearance. Results represent the mean of three independent experiments.

2.9. Analysis of A. indicum Shoot Extracts by Liquid Chromatography/Electrospray Ionization Time-of-Flight Mass Spectrometry or LC/ESI-TOF-MS. The extracts obtained by soxhlet extraction were kept. The methanolic phase was passed through C_{18} column to eliminate chlorophyll and nonpolar compounds. The sample was then passed through a 0.45 μm nylon filter before the injection into the LC-ESI-TOF-MS system. Chromatographic and mass spectrometer conditions: the separation of selected phenolic compounds was carried out using an HPLC system (Agilent 1200, Agilent technologies, Germany) equipped with a reversed phase C_{18} analytical column (2.5 × 50 mm) and 1.8 μm particle size (Zorbax Eclipse XDB-C_{18}). The mobile phase B was milli-Q water consisted of 0.1% formic acid. The mobile phase A was acetonitrile. This HPLC system was connected to a time-of-flight mass spectrometer, Agilent MSD TOF (Agilent technologies, Germany), equipped with an electrospray interface operating in positive and negative modes. In this study, some parameters were used in order to increase the possibilities of separation, detection, and characterization of phenolic compounds that are responsible for the biological activities. About the chromatographic conditions: the column temperature was maintained at 23°C, the flow rate of the mobile phase was 0.4 mL/min, and the injected sample volume was 2 μL. The optimised gradient elution was illustrated as follows: 0–10 min, 10–20% A; 10–15 min, 20–30% A; 15–25 min, 30–50% A; 25–35 min, 50–70% A; 35–40, 70–80% A; 40–65 min, which return to initial conditions. Concerning MS conditions, the capillary voltage was 3500 V, the nebuliser pressure 30 psig, drying gas 8 l/min, gas temperature 325°C, fragmentor voltage fragment 175 V, skimmer voltage 65 V, and octopole RF 750 V. LC/MS accurate mass spectra were recorded across the range 100–3000 m/z. Electrospray ionization is operated in positive mode. The data recorded was processed with MassHunter software (Germany) with accurate mass application of specific additions from Agilent MSD TOF software. UV absorption spectra were recorded online during the HPLC analysis. The DAD detector was set to a scanning range of 200–400 nm. The phenolic compounds were identified mainly by their UV data, ESI-MS spectra, and by comparing with published data.

Table 1: Phenolic contents and antioxidant activities of *A. indicum* shoot extracts. The antioxidant activities of extracts were evaluated using total antioxidant activity, antiradical activity as well as the capacity of the extract to reduce the Fe^{3+}.

Phenolic contents	Total polyphenols (mg GAE/g DW)	Total flavonoids (mg EC/g DW)	Condensed tannins (mg EC/g DW)
	19.08	11.12	1.8
Antioxidant activities	Total antioxidant activity (mg GAE/g DW)	Antiradical activity (IC_{50} μg/mL)	Reducing power (EC_{50} μg/mL)
	130	40	290

2.10. Statistical Analysis. For all plant parameters, all samples were analyzed in three replications. Data are shown as mean ± sd. A one-way analysis of variance (ANOVA) using the post hoc analyse with Duncan's test was carried out to test any significant differences at $P < 0.05$.

3. Results

3.1. Phenolic Contents and Antioxidant Activities of A. indicum Shoots. Total polyphenolic, flavonoid, and condensed tannin contents of *A. indicum* shoot extract at 18 mg/mL were estimated at the flowering stage. The evaluation of the antioxidant capacities of *A. indicum* shoots was determined by the antiradical activity against DPPH radical, the total antioxidant activity and the Fe-reducing power (Table 1). The phenolic compound content amounted at 19.97 mg GAE/g DW, whereas flavonoid and condensed tannin contents were 11.12 and 1.8 mg CE/g DW, respectively (Table 1). The total antioxidant activity (130 mg GAE/g DW) and the antiradical ability to quench the DPPH radical (IC_{50} = 40 μg/mL) of the shoot extracts were high and concomitant with a moderate Fe-reducing power (EC_{50} = 290 μg/mL).

3.2. Antiproliferative Effect of A. indicum Shoots on Caco-2 Cancer Cells. The strong accumulation of phenolic compounds in *A. indicum* may confer to this species a strong antiproliferative activity. This potential effect was evaluated using the Caco-2 colon adenocarcinoma cells. *A. indicum* shoot extracts inhibited the Caco-2 colon cancer cell growth in a dose-dependent manner (Figure 1(a)). At low concentrations (0.01–1 μg/mL), no significant effect on Caco-2 cell growth was observed. Whereas, from the concentration of 10 to 100 μg/mL, the plant extract significantly inhibited the growth of Caco-2 cells as compared to the control one. Besides, the most reduction of Caco-2 cell proliferation was about 55% using the high extract concentration (100 μg/mL).

To further assess whether the antiproliferative activities of extracts on Caco-2 cells were related to the DNA synthesis, the presence of chromatin condensation was analyzed by fluorescent microscopy using the DNA-binding fluorescent dye (DAPI) (Figure 1(b)). Control cells displayed nuclei with homogeneous chromatin distribution, whereas the shoot

Table 2: Effect of shoot extracts of *A. indicum* on the cell cycle arrest. Cells were treated with shoot extracts of *A. indicum* at the concentration of 100 μg/mL in order to check the cell cycle distribution. Cells were incubated in the absence (control) and presence of plant extracts during 72 h and then were analyzed by flow cytometry.

	Control	Shoots
G_0/G_1	42.4 ± 7.07	42.05 ± 5.16
S	17.65 ± 2.05	14.3 ± 2.69*
G_2/M	38.95 ± 5.02	42.2 ± 7.5*
Sub-G_0	1.05 ± 0.07	1.45 ± 0.21

The treatment time is 72 h. Data of three independent experiments are presented as mean ± sd.
*Statistical significance ($P < 0.05$) between treated and control cells.

extracts at 100 μg/mL reduced significantly the DNA synthesis. However, no significant apoptotic effect was observed.

3.3. Effect of Shoot Extraction on Cell Cycle Arrest Using Flow Cytometry. Shoot extract at 100 μg/mL affected the cell cycle distribution (Table 2). The G_0/G_1 and sub-G_0 phases showed stable percentages, whereas the S-phase percentage decreased from 17.65% in the control to 14.30%. Interestingly, the percentage of Caco-2 cells at the G_2/M phase was slightly higher after incubation with *A. indicum* extracts (42.2%) as compared to the control (38.95%).

3.4. Effect of A. indicum on the Mitogen-Activated Protein Kinases Involved in G_2/M Arrest. The expression of Erk1/2 and p38 MAP kinases, cyclin B1 and checkpoint kinase proteins (Chk1 and Chk2) in Caco-2 cancer cells were investigated following 72 h exposure to *A. indicum* extract (Figure 2(a)). Moreover, the relative intensities of detecting the bands of the analyzed MAP kinases were shown in Figure 2(b). The cyclin B1, protein was downregulated as compared to the control, whereas the ErK protein expression level was moderately increased by the extract. In contrary, the phosphorylated form was clearly down-regulated. The activation of Erk1/2 protein in cells treated with shoot extracts decreased as compared to the control. In fact, data showed that the ratio pErk/total Erk in cells treated by *A. indicum* dropped as a consequence of the inhibition of the activated form of Erk (pErk). Levels of regulation that contribute to stopping cell division and which involve different MAP kinases are summarised in Figure 2(b). In this context, the crude shoot extract of *A. indicum* appeared to have an upregulating effect on the level of pp38 protein expression as compared with the p38 MAP kinase. This is responsible for the downregulation of the cyclin B1 via the Cdc25c. Furthermore, the treatment with *A. indicum* extracts decreased the expression of the checkpoint kinases Chk1 and Chk2 unlike to what occurred for pChk2. The phosphorus initially attached to the Erk is transmitted to Chk1 and Chk2 MAP kinases which induce the inactivation of the CDK1/cyclin B1 complex. This is the last level of the cell cycle regulation shown after treatment.

3.5. LC-TOF-MS Identification of Bioactive Metabolites in Shoot Extracts of Arthrocnemum indicum. The analysis of the methanolic extracts of *A. indicum* by LC-ESI-TOF-MS in positive mode revealed that this halophyte plant is rich in phenolic compounds. Eight compounds were characterised and further identified by referring to the literature reporting their occurrence in the *Chenopodiaceae* family. The obtained total ion chromatogram (TIC) is illustrated in Figure 3. Furthermore, the extracted ion chromatogram (EIC) and mass spectrum (MS) of each phenolic compound are shown in Figure 4. The analysis showed the strong antioxidant activity of *A. indicum* shoot extracts. Five flavonoid compounds were identified (Figures 4(A′) to 4(F′)): 3-hydroxy-4′-methoxyflavone (m/z = 269,1452), cyanidin (m/z = 288,2690), chrysoeriol (m/z = 301,1448), quercetin (m/z = 303,2535), and luteolin (m/z = 287,2202). Moreover, shoots of *A. indicum* accumulate two phenolic acids, namely, gallic (m/z = 171,1076) and syringic (m/z = 199,1697) acids. Catechol (m/z = 111,1173) was also detected with LC-TOF-MS. All these phenolic compounds mentioned are summarised in Table 3, with their molecular formula, selected ion $[M + H]^+$, retention time (R_t), and UV data of each compound.

4. Discussion

4.1. Phenolic Contents and Antioxidant Activities of A. indicum Shoots. During the past decades, there was an increasing interest in traditional medicine and herbal products. Interestingly, extremophile plants such as halophytes appear to be useful in term of biological activity due to their substantial content in bioactive substances. This was confirmed by the present study on *A. indicum* shoot extracts since the values of total polyphenolic, flavonoid, and condensed tannin contents found were relatively high. The high content of natural polyphenol was already mentioned in tissues of the halophytes *Mesembryantimum* and *Limoniastrum* species [18]. With respect to flavonoids and tannins, these compounds contribute significantly to the total antioxidant activity of many fruits such as red grapes, vegetables, and medicinal plants such as *Nigella sativa* [26]. Shoot extracts were also characterised by an important antioxidant capacity especially against the free DPPH radical at the flowering stage. This could be partly ascribed to the strong accumulation of phenolic compounds during this specific developmental stage. Ksouri et al. [19] reported that *Tamarix gallica* flower extracts had an important total polyphenol content (135 mg GAE/g DW) which was associated with significant antiradical activity and Fe-reducing power (IC_{50} and EC_{50} values were about 2 and 45 μg/mL, resp.).

4.2. The Antiproliferative Effect of Arthrocnemum indicum Shoots. The antiproliferative effect of various concentrations of polyphenolic extracts was assessed on Caco-2 cell line. Cell proliferation was inhibited in a dose-dependent manner, the optimal concentration of the extract amounting to 100 μg/mL. The anticancer effect of natural products was already demonstrated on different cancer cell lines. For instance, Ren et al. [27] showed the antiproliferative effect

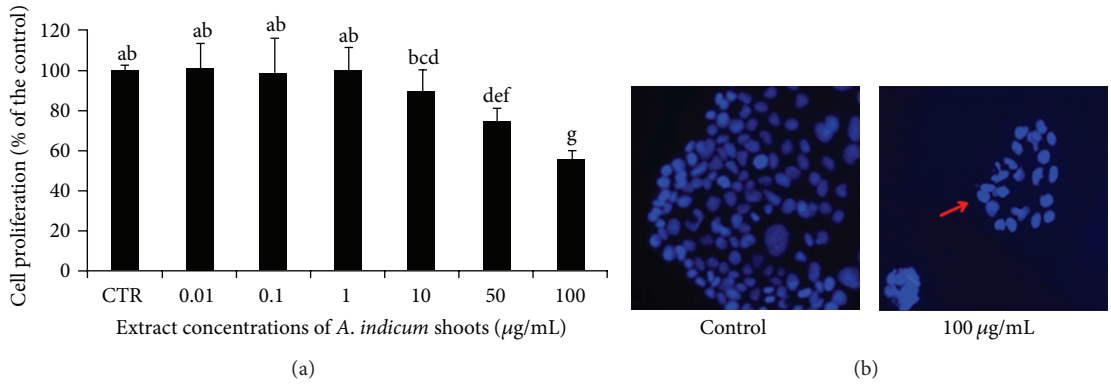


Figure 1: (a) Measurements of cell proliferation using MTT assay in human carcinoma Caco-2 cell line treated with *A. indicum* shoot extracts. Cells at 2×10^4 cells/mL were left untreated or were treated with 0.01, 0.1, 1, 10, 50, or 100 μg/mL of *A. indicum* for 72 h. Values represent the results of three independent experiments. (b) DAPI staining of Caco-2 cells treated with *A. indicum*. Cells at 2×10^4 cells/mL were incubated with 100 μg/mL of *A. indicum* for 72 h. Control cells were incubated with 0.4% DMSO. After 72 h, the nuclear morphologies of cells were examined using a fluorescent DNA-binding agent, DAPI. The DNA was analyzed using fluorescence microscopy. The arrow indicates mitotic cells with chromatin distribution. Results shown (a) and (b) are typical of 3 independent experiments.

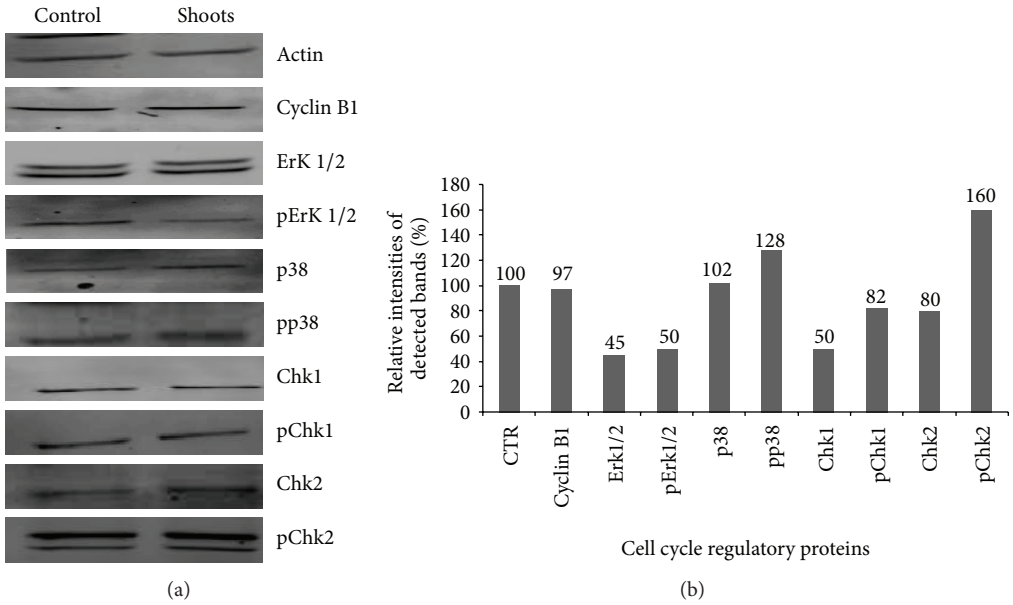


Figure 2: (a) Western analysis of the cell cycle regulatory proteins. Cells at 2×10^4 cells/mL were treated with 100 μg/mL of *A. indicum* for 72 h. After protein extraction, the same blot was incubated with the appropriate antibodies. The analyzed MAP kinases were cyclin B1, Erk1/2, pErk1/2, p38, pp38, Chk1, pChk1, Chk2, and pChk2. Results shown are typical of 3 independent experiments. (b) Relative intensities of detected bands of cyclin B1, Erk1/2, pErk1/2, p38, pp38, Chk1, pChk1, Chk2, and pChk2.

Table 3: Bioactive secondary metabolites determined by HPLC-ESI-TOF-MS in a methanol extract of *A. indicum* shoots.

Peaks	R_t (min)	λ max (nm)	$[M + H]^+$ (m/z)	Compounds (tentatively identified)	Molecular formula
1	1.616	280, 210	171.1076	Gallic acid	$C_7H_6O_5$
2	24.024	280, 255	269.1452	3-Hydroxy-4′-methoxyflavone	$C_{16}H_{12}O_4$
3	25.726	280	288.2690	Cyanidin	$C_{15}H_{11}O_6$
4	32.789	280, 240	301.1448	Chrysoeriol	$C_{16}H_{12}O_6$
5	34.169	280, 230, 260	303.2535	Quercetin	$C_{15}H_{10}O_7$
			111.1173	Catechol	$C_6H_6O_2$
6	34.939–35.260	280, 210	199.1697	Syringic acid	$C_9H_{10}O_5$
			287.2202	Luteolin	$C_{15}H_{10}O_6$

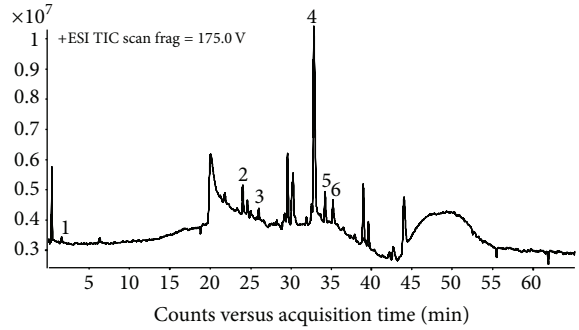


Figure 3: Total ions chromatogram (TIC) of *A. indicum* methanolic extract obtained by LC-ESI-TOF-MS. Peaks designation: (1) gallic acid, (2) 3-hydroxy-4′-methoxyflavone, (3) cyanidin, (4) chrysoeriol, (5) quercetin, (6) catechol, syringic acid, and luteolin.

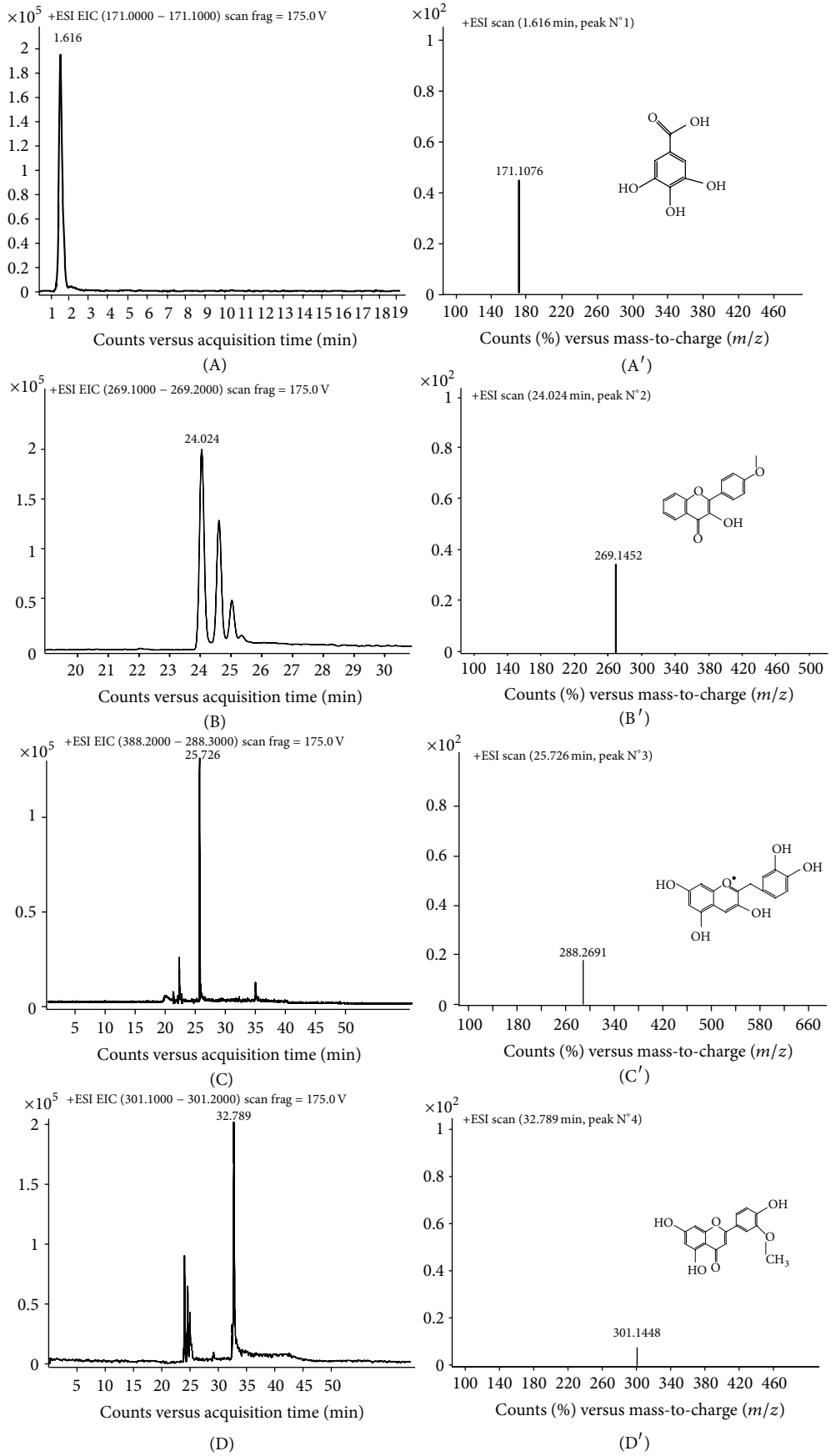
of the acetone (AEL) and methanolic (MEL) extracts from *Lethariella zahlbruckneri* on HT-29 human colon cancer cells. Both extracts of *L. zahlbruckneri* decreased viable cell number in dose- and time-dependent manners. With respect to Caco-2, phenolic compounds from apple fruit extracts (with or without skin) inhibited the proliferation of this cell line in a dose-dependent manner [21].

In order to better understand the effect of shoot extracts on cell division, the presence of chromatin condensation was analysed by fluorescence microscopy using the DNA-binding fluorescent dye DAPI. This method was already used to demonstrate the cytotoxicity and the apoptosis effects of crude extracts of *Euchresta formosana* radix in the human hepatocellular carcinoma Hep3B cell line [28]. In our study, the control cells showed nuclei with homogeneous chromatin distribution, whereas treatment with 100 μg/mL of extracts decreased the chromatin amount. Hence, shoot extracts had a marked effect on the DNA synthesis. The decrease of DNA biosynthesis which is an indicator for the decrease of the number of cells during treatment provides another argument for the antiproliferative effect of *A. indicum* shoot extracts on Caco-2 cells. In the present study, no apoptosis effect could be observed using the fluorescence microscopy by DAPI staining. Therefore, further experiments like the investigation of the cell cycle distribution are needed to highlight the mechanisms involved in the anticancer activities of those compounds.

4.3. Effect of Shoot Extraction on Cell Cycle Arrest Using Flow Cytometry. Given that cell division control is the major regulatory mechanism of cell growth, the analysis of the cell cycle is a novel and relevant approach for cancer control and eradication [7]. Our findings showed that Caco-2 cells were blocked at the G_2/M phase following 72 h exposure to *A. indicum* shoot extracts at 100 μg/mL. This was already observed in chronic myeloid leukemia (K562) cells treated with *Stellera chamaejasme* extract [6]. The same effect on K562 cells was also reported using a novel and synthetic anticancer agent, the enediyne derivative THDA [29]. Moreover, the role of flavonoid compounds such as 2′-nitroflavone was mentioned for a similar effect on HeLa human cervical carcinoma cells [30].

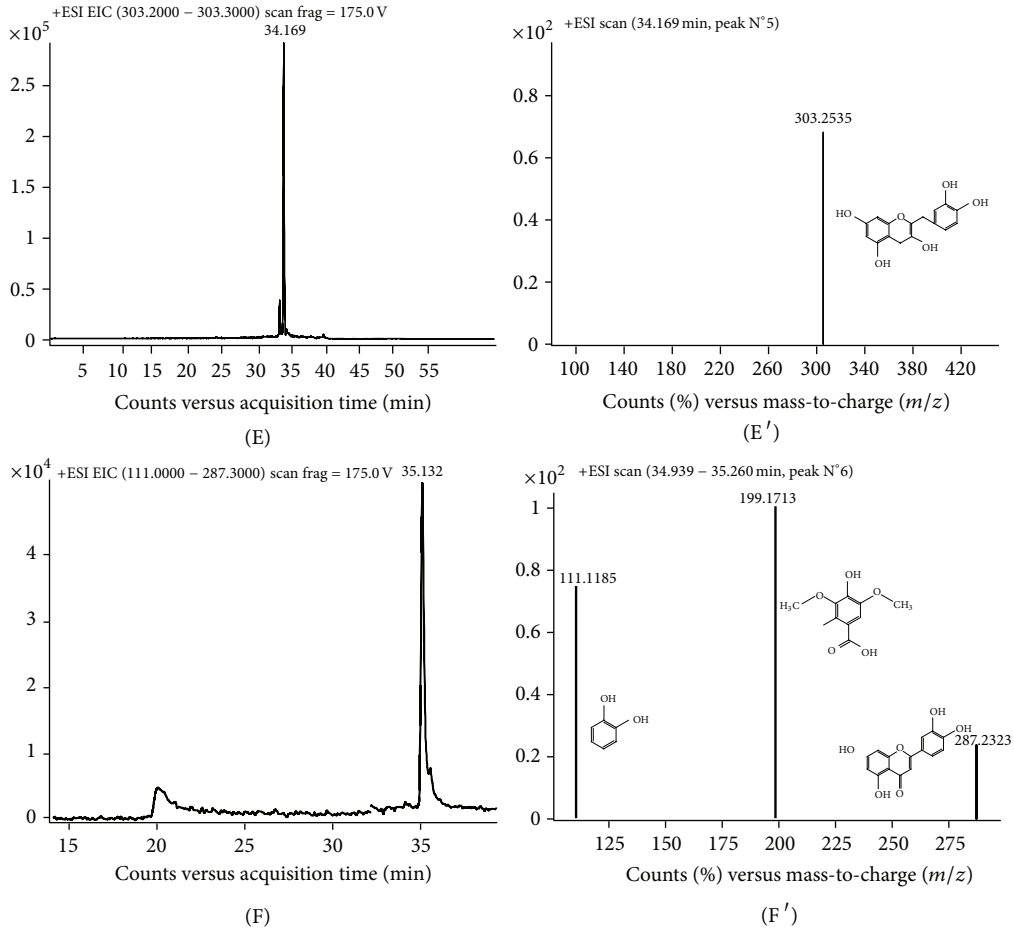
4.4. Effect of Proteins Involved in the G_2/M Arrest. Cell division is a complex phenomenon that is regulated by a number of protein kinases whose role is the transcription of genes essential for entry into division. These mitogen-activated proteins or MAP Kinases play an essential role in the initiation, the progression, and the coupling of these phases [13]. Our study which aimed at better understanding of the molecular mechanisms of G_2/M phase arrest induced by *A. indicum* showed that extracts at 100 μg/mL blocked the Caco-2 cell cycle at G_2/M phase. Moreover, *A. indicum* had an effect on the expression of specific cell cycle-associated protein kinases (cyclin B1, p38, Erk1/2, Chk1, and Chk2) occurring together with the changes in cell cycle distribution. *A. indicum* shoot extracts have a downregulating effect on the expression of cyclin B1 protein. It is assumed that Cdc25c plays a role in the regulation of the Cdk1/cyclin B1 complex [16]. Shoot extracts of *A. indicum* had an upregulating effect on the level of pp38 protein but not on the pErk one. The role of p38 as a key protein in the regulation of cell division was already mentioned in previous studies as an important protein implicated in the suppression of tumorigenesis [15, 17]. The expression of checkpoint kinases Chk1 and Chk2 was also affected, both of these proteins being involved in the anticancer effect in relation with the cell cycle arrest [14]. Thus, all these analyzed protein expressions show for the first times a mechanism related to Caco-2 cancer cells in response to *A. indicum* treatment. The identification of such biological compounds from this plant is needed to clarify the biological effect of this medicinal plant.

4.5. LC-TOF-MS Identification of Bioactive Secondary Compounds. In the *Chenopodiaceae* family, some phenolic compounds are abundant. In the present study, gallic acid, 3-hydroxy-4′-methoxyflavone, cyanidin, chrysoeriol, quercetin, catechol, syringic acid, and luteolin were characterised from the aerial parts of *A. indicum*. At 1.616 min (Figure 4(A)), the ion $[M+H]^+$ found in positive mode, likely, corresponds to gallic acid with a molecular formula $C_7H_6O_5$ (Figure 3, Peak no. 1) [31]. At 24.024 min (Figure 4(B)), ion 3-hydroxy-4′-methoxyflavone with the formula $C_{16}H_{12}O_4$ (Figure 3, Peak no. 2) was already found in some African plants [32]. Cyanidin (Figure 3, Peak no. 3) was identified with the molecular formula $C_{15}H_{11}O_6$ detected by the LC-TOF/MS at 25.726 min (Figure 4(C)). However, the compound detected at 32.789 min (Figure 4(D)) was identified as chrysoeriol with the molecular formula $C_{16}H_{12}O_6$ [33] (Figure 3, Peak no. 4). Also, molecular ion detected at 34.169 min (Figure 4(E)) corresponds to quercetin $C_{15}H_{10}O_7$ (Figure 3, Peak no. 5) [34]. As shown in the TIC (Figure 3, Peak no. 6), three ions were detected at 34.939–35.260 min (Figure 4(F)). As a function of previous studies, the first one corresponds to catechol ($C_6H_6O_2$) [35], the second identified as syringic acid ($C_9H_{10}O_5$) [36], and the last one represents luteolin ($C_{15}H_{10}O_6$) according to Liu et al. [37].



(a)

Figure 4: Continued.



(b)

Figure 4: Extract ions chromatogram/mass spectra of each protonated molecule (positive mode): gallic acid ((A)/(A′)), 3-hydroxy-4′-methoxyflavone ((B)/(B′)), cyanidin ((C)/(C′)), chrysoeriol ((D)/(D′)), quercetin ((E)/(E′)), and catechol, syringic acid, and luteolin ((F)/(F′)).

A. indicum shoot extracts produced high antioxidant activities and high antiproliferative activities. This may be explained by the nature of *A. indicum* phenolic amounts. The antioxidant effects of some natural bioactive molecules found in shoot extracts of *A. indicum* as syringic acid, chrysoeriol, and quercetin were already demonstrated in previous studies [38, 39]. Moreover, the methoxyflavonoid and chrysoeriol, selectively inhibit the formation of a carcinogenic estrogen metabolite in MCF-7 breast cancer cells [40]. In addition, luteolin, quercetin, and gallic acid were known by their significant antiproliferative effect [4, 41, 42]. According to Zhang et al. [43], flavonoids such as flavones (luteolin) and flavonols (quercetin) have an important cytotoxic effect on human oesophageal adenocarcinoma cell line (OE33) inducing a cell cycle arrest at the G_2/M phase. The antioxidant and antiproliferative effects of *A. indicum* extracts could also be explained by the possibility of synergy between components. This has already been demonstrated using HL-60 cells treated with Tunisian Gerboui olive leaf extracts [44].

In conclusion, *A. indicum* may be useful as a candidate in the treatment of the colon cancer in a specific manner. In fact, the high anticancer and antioxidant activities found in shoots of this halophyte could be ascribed to the high total polyphenol content, whereas the significant antiproliferative effect could be explained by the cell cycle arrest on G_2/M phase determined by flow cytometry. These activities seem to be related to the accumulation of phenolic compounds in *A. indicum*. In this context, eight metabolites were characterised by LC-TOF-MS analysis.

Conflict of Interest

There is no potential conflict of interests involved with this work.

Acknowledgments

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Review Article

Loranthus micranthus Linn.: Biological Activities and Phytochemistry

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Loranthus micranthus Linn. is a medicinal plant from the Loranthaceae family commonly known as an eastern Nigeria species of the African mistletoe and is widely used in folkloric medicine to cure various ailments and diseases. It is semiparasitic plant because of growing on various host trees and shrubs and absorbing mineral nutrition and water from respective host. Hence, the phytochemicals and biological activities of *L. micranthus* demonstrated strong host and harvesting period dependency. The leaves have been proved to possess immunomodulatory, antidiabetic, antimicrobial, antihypertensive, antioxidant, antidiarrhoeal, and hypolipidemic activities. This review summarizes the information and findings concerning the current knowledge on the biological activities, pharmacological properties, toxicity, and chemical constituents of *Loranthus micranthus*.

1. Introduction

Loranthus micranthus (*L. micranthus*) as member of the Loranthaceae family is the eastern Nigeria species of the African mistletoe. Mistletoes are the semiparasitic plants because they normally grow on various host trees and shrubs and they are dependent on their respective host for mineral nutrition and water, although they produce their own carbohydrates through photosynthesis [1]. Mistletoe was described as "an all purpose herb" due to its rich traditional uses and it has been widely used in ethnomedicine for various purposes, including antihypertensive, anticancer, antispasmodic, and antidiabetic, and for treatment of epilepsy, headache, infertility, menopausal syndrome and rheumatism [2, 3]. Previous studies demonstrated that composition and hence biological activities of mistletoe are dependent on harvesting period and host tree species [4–7]. Nigeria has wide distribution of mistletoes with different local names that depend on the area where they occur. *Loranthus micranthus* represents Eastern Nigeria mistletoes that mostly grow in the southeastern region of the country. It grows on various host trees including *Persia americana, Baphia nitida, Kola acuminata, Pentaclethra macrophylla,* and *Azadirachta indica* [8, 9]. *L. micranthus* has been widely used in folk medicine as antimicrobial, antihypertensive, anticancer, and antidiabetic agent, for treatment of headache, infertility, epilepsy, cardiovascular diseases, menopausal syndrome, agglutination, and rheumatism, and also in conditions that generally require immunomodulatory. Some of these ethnomedicinal uses have already been supported and acclaimed by several

investigations [1, 2, 10, 11]. In Nigeria and South Africa, *L. micranthus* has been widely used as ethnomedicine for treatment of hypertension, diabetes, and schizophrenia and as an immune system booster [10].

2. Phytochemistry

Extensive phytochemical evaluations of *L. micranthus* extracts demonstrated the presence of various phyto-constituents and compounds. Crude methanolic extract from leaves of *L. micranthus* harvested from *P. americana* was found to possess terpenoids, steroids, oils, proteins, resins, flavonoids, tannins, saponins, alkaloids, reducing sugar, acidic compounds, glycosides, and carbohydrates. Alkaloids showed the highest presence in abundance. The weakly acidic fraction analysis isolated from aqueous methanolic extract of leaves of *L. micranthus* showed the presence of terpenoids, steroids, acidic compounds, flavonoids, and carbohydrate. Lower rate of flavonoids and carbohydrates elicited in comparison with methanolic extract, while the amount of terpenoids, acidic compounds, and steroids remained unchanged [28]. Chemical composition of *L. micranthus* was found to be seasonal dependent. During April, the onset of rainy season, methanolic extract of the leaves harvested from *P. Americana* showed higher concentrations of carbohydrates, acidic compounds, steroids, and alkaloids compared to July, the time for peak of rainy season. Interestingly, Tannins, saponins, glycosides, and reducing sugars were not found in July samples. However, higher amounts of flavonoids and oils were detected in July samples compared to April ones [29]. Iwalokun and colleagues [30] have investigated the phytochemicals such as *n*-butanol, chloroform, ethyl acetate, and water fractions of the methanolic extract of *L. micranthus* leaves of Kola nut tree (*K. acuminata*). Moderate and high levels of steroids and terpenoids were detected in *n*-butanol fraction, while phenolics, reducing sugars, and tannins were detected in all fractions together with moderate level for phenolics and tannins in chloroform fraction. Indeed, flavonoids and saponins were only present in ethyl acetate and water fractions, respectively. They have demonstrated that terpenoids were present in low-to-moderate levels in chloroform and water fractions, while they were not detected in ethyl acetate fraction. Phytochemical studies on *L. micranthus* leaves harvested from six different host trees, namely, *P. americana, B. nitida, K. acuminata, P. macrophylla, A. indica,* and *Irvingia gabonensis,* revealed that alkaloids are preponderant in the extracts of *K. acuminata, P. Americana,* and *I. gabonensis*. Moreover, the phytochemical constituent host dependency was also shown [31, 32]. A study on petroleum ether extract of *L. micranthus* leaves parasitic on *P. americana* harvested at different seasons (January, April, July, and November) showed only presence of alkaloids in April and July and proved harvesting period dependency in phytochemicals of *L. micranthus* [33]. In several studies attempting to identify the active compounds responsible for various biological activities of *L. micranthus*, especially immunomodulatory activity, a variety of compounds (Table 1) were isolated and their structures were characterized (Figure 1).

3. Biological Activities

Fractions, pure compounds, and crude extracts of plants are precious and crucial resources for effective molecules in treatment of different diseases and ailments in animal and human [34]. *L. micranthus* has been found to possess antidiabetic, antimicrobial, antihypertensive, hypolipidemic, antioxidant, antidiarrhoeal, and immunomodulatory activities. A study by Edem and Usoh [35] demonstrated that the use of *L. micranthus* is safe and without adverse biochemical effects or hepatocellular damages in rats. They have administered different doses of the water extract of *L. micranthus* leaves from 275 to 827 mg/kg for 21 days on male albino Wistar rats. Blood samples analysis did not reveal any significant changes in the level of protein, urea, glucose, bilirubin, cholesterol, alkaline phosphatase, and aspartate transaminase. However, significant reduction was observed in the level of alanine transaminase enzyme by using 551 and 827 mg/kg of the extract. However, an *in vitro* study on aqueous leaf extract of *L. micranthus* indicated cytotoxic, genotoxic, and mitodepressive effects of that extract against *Allium cepa* root cells especially at dose beyond pharmacological range [36]. They showed a significant inhibition of root growth with an effective concentration (EC_{50}) of 28.2 mg/mL. The extract revealed dose-dependent decrease in mitotic index by 2.4–27.4% using a range from 5 to 40 mg/mL of the extract except at 2.5 mg/mL in which 11.9% elevation was reported compared to 50% decrease for sodium azide used at 100 μg/mL as positive control. Hence, for safety reasons, using the lower concentrations of *L. micranthus* for human phytomedicine in prolonged use coupled with *in vivo* genotoxic tests is suggested [36].

3.1. Immunomodulatory Activity. Stimulation of human immune system has been identified as a possible pathway to inhibit the progression of some diseases without eliciting adverse side effects [37, 38]. However, immune system stimulation is a desired response if overall process culminates with cure or faster convalescence from sickness [39, 40]. One folkloric use of *L. micranthus* is due to its ability to enhance the immune system with concomitant quicker convalescence [10]. An *in vivo* study on aqueous-methanol extract from *L. micranthus* leaves harvested from five different host trees has investigated the immunomodulatory activity in rat and mice models, including the cyclophosphamide-induced myelosuppression (CIM) test, the humoral mediated antibody titration (AT) test, total and differential leukocyte count (TLC and DLC), and the cellular mediated delayed-type hypersensitivity reaction (DTHR) test [10]. According to the results, the extracts (with overall order of activity from *K. acuminate* > *Citrus* spp. > *P. Americana* > *Parkia biglobosa* > *P. macrophylla*) were found to be potent and safe complementary or alternative medicines to cure the immunodeficiency conditions without any toxicity (LD_{50}

Table 1: Phytochemistry and bioactivity of compounds isolated from *L. micranthus*.

Plant part	Compound	Code	Chemical Category	Biological activity	Host tree	Reference
Leafy twigs	Linamarin gallate	1	Phenolics glycoside	Antioxidant activity	*Hevea brasiliensis*	[12]
	Walsuraside B	2	Phenolics glycoside	Antioxidant activity	*Hevea brasiliensis*	[12]
	Catechin	3	Polyphenol	Antioxidant activity	*Hevea brasiliensis*	[13]
	Epicatechin	4	Polyphenol	Antioxidant activity	*Hevea brasiliensis*	[14]
	Epicatechin 3-O-gallate	5	Polyphenol	Antioxidant activity	*Hevea brasiliensis*	[15]
	Epicatechin 3-O-(3-O-methyl) gallate	6	Polyphenol	Antioxidant activity	*Hevea brasiliensis*	[16]
	Epicatechin 3-O-(3,5-O-dimethyl) gallate	7	Polyphenol	Antioxidant activity	*Hevea brasiliensis*	[17]
	Epicatechin 3-O-(3,4,5-O-trimethyl) gallate	8	Polyphenol	Antioxidant activity	*Hevea brasiliensis*	[18]
	Quercetin 3-O-β-D-glucopyranoside	9	Polyphenol	Antioxidant activity	*Hevea brasiliensis*	[19]
	Rutin	10	Polyphenol	Antioxidant activity	*Hevea brasiliensis*	[20]
	Peltatoside	11	Polyphenol	Antioxidant activity	*Hevea brasiliensis*	[21]
Leaves	-(-) catechin-7-O-rhamnoside	12	Polyphenol	Immunomodulatory and antioxidant activity	*Kola acuminata*	[22]
	-(-) catechin-3-O-rhamnoside	13	Polyphenol	Immunomodulatory and antioxidant activity	*Kola acuminata*	[22]
	4′-methoxy-catechin-7-O-rhamnoside	14	Polyphenol	Immunomodulatory and antioxidant activity	*Kola acuminata*	[22]
	7β, 15α-dihydroxy-lup-20(29)-en-3β-O-palmitate	15	Triterpenoid ester	Immunomodulatory activity	*Kola acuminata*	[23]
	7β, 15α-dihydroxy-lup-20(29)-en-3β-O-stearate	16	Triterpenoid ester	Immunomodulatory activity	*Kola acuminata*	[23]
	7β, 15α-dihydroxy-lup-20(29)-en-3β-O-eicosanoate	17	Triterpenoid ester	Immunomodulatory activity	*Kola acuminata*	[23]
	stigmast-7,20(21)-diene-3β-hydroxy-6-one	18	Steroid	Immunomodulatory activity	*Kola acuminata*	[24]
	3β-hydroxystigmast-23-ene (stigmast-23-en-3β-ol)	19	Steroid	Immunomodulatory activity	*Kola acuminata*	[24]
	Lupeol	20	Triterpenoid	Immunomodulatory activity	*Kola acuminata*	[25]
	Lupinine	21	Alkaloid	Immunomodulatory activity	*Kola acuminata*	[26, 27]
	Loranthoic acid	22	Steroid	Immunomodulatory activity	*Kola acuminata*	[26, 27]

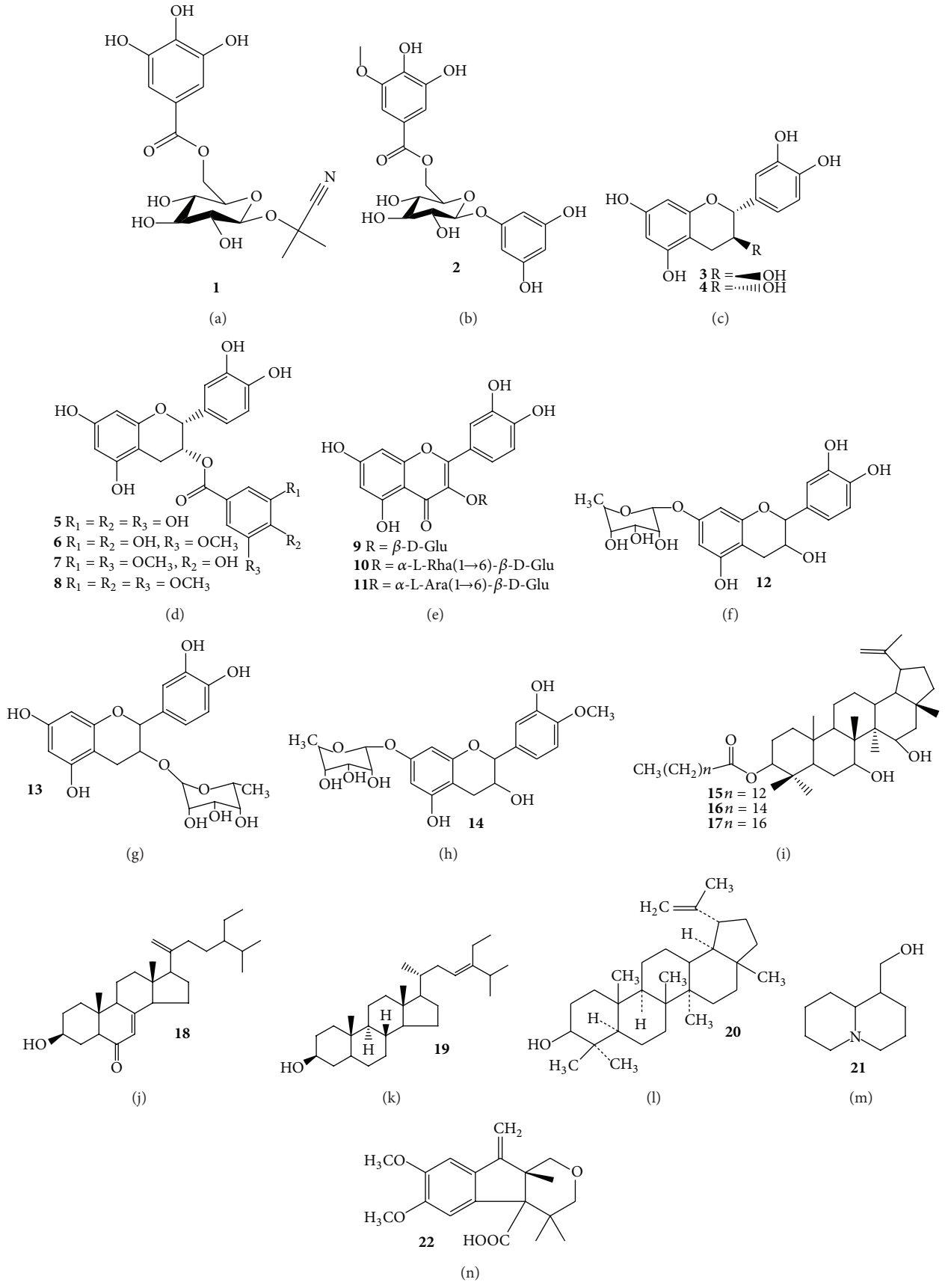

Figure 1: Chemical structure of chemical compounds isolated from *L. micranthus*.

values > 5000 mg/kg for acute toxicity tests). Leaves extract parasitic on *K. acuminate* at a dose of 200 mg/kg caused 139.69% stimulation of total leukocyte in mice compared to 75.35% increase for levamisole (25 mg/kg) as a standard immunostimulatory drug. At dose of 100 mg/kg, it also showed 175% stimulation on DTHR in immunocompetent mice compared to 122.50% increase for levamisole as positive control. 71.35% and 81.53% of primary and secondary stimulation on antibody titration in rats at a dose of 100 mg/kg of leaves extract also exhibited the significant immunomodulatory effect compared to 24.50% and 0.40% of primary and secondary stimulation for levamisole (25 mg/kg), respectively [10, 41]. Results from another study by the same group showed that ethanol and *n*-hexane leaves extracts parasitic on *P. americana* at doses from 100 to 400 mg/kg also exhibited a dose-dependent immunomodulatory activity assessed by DTHR and CIM models in mice. Results showed over 170% of stimulation for both extracts at 400 mg/kg dose [42]. The immunomodulatory analysis of five different extracts of *L. micranthus* leaves, parasitic on *K. acuminata*, namely, *n*-hexane, chloroform, ethyl acetate, acetone, and methanol by DTHR test in experimental mice and their phytochemical evaluation of the fractions, demonstrated that the most active fractions were either mainly terpenoidal, flavonoidal, or steroidal [43]. The results also confirmed the most significant immunostimulatory effects for chloroform extract with 311.11% and 122.22% enhancement in stimulation using 250 and 500 mg/kg of the extract, respectively. The compounds **12**–**14** at dose of 100 μg/mL showed increasing effects on C57BL/6 mice splenocytes proliferation with 91.49%, 95.17%, and 94.23% stimulation values, respectively, compared to 16.09% stimulation for 10 μg/mL of lipopolysaccharide (LPS) as positive control. However, compounds **12**, **13** and **14** exhibited moderate stimulatory effect on CD69 molecule expression [22]. The compound **15** with dose of 100 μg/mL exhibited a weak immunostimulatory activity by inducing 24.44% and 86.98% stimulation of mice splenocytes proliferation and early activation of CD69 molecule, respectively. In addition, it also showed a nonsignificant effect on IL-8 receptor expression [44]. The steroids compounds of **18** and **19** at a dose of 100 μg/mL exhibited significant immunostimulatory activity on the C57BL/6 mice splenocytes with 46% and 43% stimulation, respectively, compared to 7.69% increase for the negative control, although CD69 expression assay revealed minimal stimulation. The compounds **16** and **17** with the same concentration showed weaker stimulations of 30% and 29%, respectively, on the C57BL/6 mice splenocytes [24]. Also, a mild immunostimulatory activity was observed for compound **20** when it was tested on C57BL/6 splenocytes [25]. However, 69.84% and 56.34% stimulation elicited from compounds **21** and **22** at a dose of 25 μg/mL for proliferation analysis on mice splenocytes (C57BL/6) compared to 7.58% value for unstimulated control. The CD69 expression assay also exhibited significant proliferation values of 2.71% and 2.31% for compounds of **21** and **22**, respectively [27].

3.2. Antidiabetic Activity. Diabetes mellitus is one of the prevalent and serious chronic diseases around the world [45]. Therefore, finding the promising ways to improve the quality of life for diabetic patients and preventing or reversing diabetic complications necessitate investigations among the arsenal of herbs [46]. Osadebe and colleagues [32] have studied the anti-diabetic activity of the methanolic extracts of leaves of *L. micranthus* parasitic on *P. americana, B. nitida, K. acuminata, P. macrophylla,* and *A. indica*. The extracts were found to possess significant dose-dependent antihyperglycemic effects in alloxan-induced diabetic albino and normoglycemic rats, respectively. The maximum activity of the methanolic extract of *L. micranthus* (400 mg/kg) harvested from *P. americana* on alloxan-induced diabetic rats showed 82.59% reduction of blood sugar level at 24 h after administration determined by *o*-toluidine spectrophotometric method which is statistically comparable with the 83.34% of reduction for glibenclamide as a positive control. Methanolic extracts of *L. micranthus* from five different host trees did not show any toxicity according the acute toxicity tests in mice (LD_{50} values of 11650, 11650, 5900, 5900, and 5900 mg/kg for *P. americana, B. nitida, K. acuminata, P. macrophylla,* and *A. indica,* resp.). The leaves of *L. micranthus* parasitic on *K. acuminata*, *A. indica,* and *B. nitida* showed more significant antihyperglycemic activity among the other host trees investigated. The results demonstrated that the antidiabetic effect of the extract was found to be dependent on the host plant species. The weakly acidic fraction of the aqueous methanol extract of the leaves of *L. micranthus* parasitic on *P. americana* revealed anti-diabetic activity in alloxan-induced diabetic rats. The low-acidic fraction at the dose of 400 mg/kg reduced 66.60% of blood sugar level of alloxan-induced diabetic rats at 24 hours after administration [28]. However, Osadebe and colleagues [29] have studied the seasonal variation for the anti-diabetic effect of the aqueous methanolic extract of the leaves of *L. micranthus,* parasitic on *P. americana,* in alloxan-induced diabetic rats. The study demonstrated that anti-diabetic effect of the extract is seasonal and dose dependent with the highest activity being at the peak of the rainy season. The results revealed 39.2% and 47.5% fasting blood sugar reduction after 6 hours consumption of 400 mg/kg of April and July samples, respectively, with 8.3% difference due to effect of seasonal variation on chemical content of leaves. Higher concentration for flavonoids in peak of the rainy season compared to the onset of rainy season could be responsible for the observed higher anti-diabetic activity in July. However, there is no data available for the particular bioactive compound(s) from *L. micranthus* with known mechanism for anti-diabetic activity. Therefore, this is an open area for the future research around this plant. Uzochukwu and Osadeb [47] have studied a comparative evaluation of antidiabetic activities of crude methanolic extract and flavonoids extract of *L. micranthus* harvested from *K. acuminata* in alloxan-induced diabetic rats. Results revealed that flavonoids extract (200 mg/kg) showed significant anti-diabetic effect within one hour of administration, while the methanolic extract (200 mg/kg)

showed the significant antidiabetic effect within three hours of administration. In addition, phytoconstituents of other members of Loranthaceae plants possessing antihypertensive activity are interestingly similar to *L. micranthus* [48–50].

3.3. Antimicrobial Activity. Using medicinal plants as antimicrobial remedy has a long history in both developed and developing countries. In addition, in contemporary medicine because of unreasonable and indiscriminate consumption of antimicrobial drugs, the infectious microorganisms have developed resistance. Therefore, controlling the existing infectious diseases necessitates new alternative antimicrobial drug regimens [51]. Osodebe and Ukwueze have presented the wide range of data regarding the antimicrobial activities of *L. micranthus* [31]. A study on different extracts of *L. micranthus* leaves harvested from *K. acuminata* such as methanolic, ethanolic, chloroform, and petroleum ether extracts demonstrated the antibacterial activities for all tested extracts against *Bacillus subtilis, Escherichia coli,* and *Klebsiella pneumonia,* while only petroleum ether extract exerted antifungal activity against *Aspergillus niger* and *Candida albicans* with the MIC of 4.30 and 1.73 mg/mL, respectively, and no activity against *Klebsiella pneumonia*. The methanolic extract exhibited the most potent antibacterial effect against *Bacillus subtilis* and *Escherichia coli* with MIC of 1.58 and 1.48 mg/mL, respectively, compared to the other tested extracts [52]. The ethyl acetate fraction of crude petroleum ether extract of *L. micranthus* leaves parasitic on *K. acuminata* also showed inhibitory activities against *Candida albicans* and *Bacillus subtilis* [53]. A comparative investigation on antimicrobial activities of *L. micranthus* leaves and its phytochemicals from six different host trees, including *P. americana, B. nitida, K. acuminata, P. macrophylla, A. indica*, and *I. gabonensis,* indicated the relative significant antibacterial activities for *L. micranthus* parasitic on *K. acuminata* and *P. americana*. Alkaloids in these three species were found to be most abundant. Preponderance of alkaloids in these species could be responsible for the marked antimicrobial activities. The leaves extract parasitic on *P. Americana* exerted more potent antibacterial activity against *Pseudomonas aeruginosa* compared to amoxicillin, while the extracts from *A. indica, P. macrophylla,* and *I. gabonensis* exhibited stronger antibacterial activity against *Staphylococcus aureus* in comparison with amoxicillin [31]. Effect of different harvesting seasons (January, April, July, and November) on antimicrobial activity of petroleum ether extracts from *L. micranthus* leaves, parasitic on *P. americana,* and its phytochemicals, also demonstrated seasonal dependency. Alkaloids as one of the major groups of antibacterial candidates was only found in July and April. Antibacterial activity against *Salmonella typhi* and *Bacillus subtilis* was markedly lower in January samples compared to the extracts from the other months' samples [33, 54]. In addition, no significant antifungal activity for methanolic extracts of *L. micranthus* leaves harvested from *K. acuminata*, *P. Americana,* and *I. gabonensis* was demonstrated against *A. niger* (MIC > 3 mg/mL) and *C. albicans* (MIC > 4 mg/mL) compared to ketoconazole (MIC < 1 mg/mL) as an approved antifungal agent [55]. To sum up, it has been proven that leaf extracts of *L. micranthus* elicited significant antibacterial activity against *B. subtilis, P. aeruginosa, E. coli,* and *Staph. aureus* without significant antifungal activity [56].

3.4. Antihypertensive Activity. Deaths because of hypertension arise out of cardiovascular and cerebrovascular complications including cardiac arrest, stroke, myocardial infarction, congestive heart failure, and end-stage renal disease [57]. Early detection and commencement of treatment are vital for prevention and delaying the aftermaths and enhance the chance of longer life for afflicted patients [58]. In the last few decades, plants have still remained as a rich source for efficacious, safe, and cost-effective antihypertensive drugs [59]. *L. micranthus* is one of the plants identified with antihypertensive activity for humans and animals in sub-Saharan Africa [60]. Aqueous extract of *L. micranthus* (1.32 g/kg per day) exhibited hypotensive effect on normotensive and spontaneous hypertensive rats [61]. A noteworthy reduction in the mean arterial pressure (MAP) was obtained in both groups of rats without effect on the urinary flow rate or the respiratory rate. In addition, the level of total cholesterol exhibited significant reduction on days 6, 7, and 8 [61]. Indeed, methanolic extract of leaves of *L. micranthus* demonstrated a dose-dependent inhibition of blood pressure increase in adrenaline-induced hypertensive rat. Iwalokun and colleagues [30] studied the activity of *n*-butanol, chloroform, ethyl acetate, and water fraction of the methanolic extract of *L. micranthus* leaf harvested from *K. acuminata* on pressor-induced contraction of rat aorta smooth muscles. *N*-Butanol fraction showed the highest dose-dependent inhibitory activity (EC_{50} = 0.65 mg/mL and smooth muscle relaxation of 75.2%) on rat aorta precontracted with norepinephrine and KCl, followed by decreasing order by water, chloroform, and ethyl acetate fractions. The same order of activity was observed in the ability of these fractions to reduce cardiac arginase with 11.7% reduction for *n*-butanol fraction and to raise serum nitric oxide with 55% elevation for *n*-butanol fraction in mice orally administrated 250 mg/kg of fractions for 21 days. Arginase was found to be a diagnostic indicator for cardiovascular diseases including hypertension [62]. Enhanced activity of nitric oxide is a critical factor to reduce the vascular resistance and blood pressure that increased in hypertensive rats and humans [63, 64]. Cardiac arginase reduction, vasorelaxation, antiatherogenic events, and nitric oxide (NO) elevation were found to be responsible for antihypertensive activity of *L. micranthus.* Moderate and high abundance of steroids and terpenoids in *n*-butanol fraction strongly suggested that these phytoconstituents could be responsible for observed vasorelaxant and antiatherogenic activity of *n*-butanol fraction. Iwalokun and colleagues [30] have reviewed the possible mechanisms of antihypertensive activity of *L. micranthus.*

3.5. Antioxidant Activity. The role of free radicals in many diseases has been proven by recent developments in biomedical sciences. Cellular damage caused by free radicals is possibly responsible for many degenerative diseases including heart disease, cancer, brain dysfunction, and immune system

decline [65]. Phenolic compounds are potent scavengers of free radicals [66]. The antioxidant activity of compounds **1**–**11** isolated from methanol extracts of *L. micranthus* leafy twigs was investigated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. All the tested compounds revealed significant antioxidant effect (IC_{50} = 23.8–50.1 μM) compared to chlorogenic acid as a positive control (IC_{50} = 67.9 μM). Compounds **10** and **11** exhibited the most significant antioxidant activity [12]. The results of DPPH assay on compounds **12**–**14** revealed significant antioxidant potentials with EC_{50} of 55.42, 58.45, and 59.71 mg/mL, respectively, compared to ascorbic acid as a positive control with EC_{50} = 17.6 mg/mL [22].

3.6. Antidiarrhoeal Activity. Diarrhoea due to the risk of sever potentially fatal dehydration can be a serious complication in infants and elderly people [67]. About 2.2 million people, mostly infants and children below 5 years, are victims of diarrhea annually [68]. Because of the adverse effects of some existing antimotility medicines after prolonged use, many studies have been done for an alternative remedy among traditional medicinal herbs [69, 70]. *In vivo* study on methanolic extract of *L. micranthus* leaf harvested from six different host trees, including *P. americana, B. nitida, K. acuminata, P. macrophylla, C. sinensis,* and *I. gabonensis,* indicated antimotility effect in rats with castor-oil-induced diarrhoea. Methanolic extract of *L. micranthus* parasitic on *P. macrophylla* (200 mg/kg) showed the most significant decrease on defecation (93.33%) 4 hours after administration compared to atropine sulphate (2 mg/kg) as a positive control (80%). It also significantly inhibited gastrointestinal transit by 67.6% which is more than atropine sulphate (26.4%) [9].

3.7. Hypolipidemic Activity. The serum lipid analysis in mice orally was administered with 250 mg/kg of *n*-butanol, chloroform, ethyl acetate, and water fractions of the methanolic extract of *L. micranthus* harvested from *K. acuminata* for 21 days indicated with decrease in cholesterol and triglyceride with the highest activity for *n*-butanol fraction. Results demonstrated lower total cholesterol, triglycerides (TAG), and LDL-cholesterol levels particularly for *n*-butanol and water fractions after 21 days without significant changes for HDL-cholesterol. On day 21, *n*-butanol fraction significantly reduced TAG level (138.5 ± 3.4 versus 152.8 ± 0.6 mg/dL). Total cholesterol and LDL-cholesterol levels also reduced from 114.3 ± 3.5 mg/dL to 91.9 ± 0.4 mg/dL and from 42.2 ± 4.3 mg/dL to 23.7 ± 0.9 mg/dL by *n*-butanol fraction on the day 21, respectively [30].

4. Conclusion

The overview of scientific investigations on *L. micranthus* showed various biological activities for this plant. The phytochemical constituents and the activity of the medicinal values of *L. micranthus* are strongly dependent on harvesting season and host species trees. Phytochemicals and compounds isolated from *L. micranthus* are in interest for the further investigations towards application of this plant as anti-diabetic, antibacterial, antihypertensive, hypolipidemic, antioxidant, antidiarrhoeal, and immunomodulatory medicines. Further *in vivo* genotoxic tests of this plant can be also beneficial for the safety approval for therapeutic applications.

Conflict of Interests

All authors declared that there is no actual or potential conflict of interests including any financial, personal, or other relationships with other people or organizations that could inappropriately influence or be perceived to influence their work.

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Research Article

Activation of MITF by Argan Oil Leads to the Inhibition of the Tyrosinase and Dopachrome Tautomerase Expressions in B16 Murine Melanoma Cells

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Argan (*Argania spinosa* L.) oil has been used for centuries in Morocco as cosmetic oil to maintain a fair complexion and to cure skin pimples and chicken pox pustules scars. Although it is popular, the scientific basis for its effect on the skin has not yet been established. Here, the melanogenesis regulatory effect of argan oil was evaluated using B16 murine melanoma cells. Results of melanin assay using B16 cells treated with different concentrations of argan oil showed a dose-dependent decrease in melanin content. Western blot results showed that the expression levels of tyrosinase (TYR), tyrosinase-related protein 1 (TRP1), and dopachrome tautomerase (DCT) proteins were decreased. In addition, there was an increase in the activation of MITF and ERK1/2. Real-time PCR results revealed a downregulation of *Tyr*, *Trp1*, *Dct*, and *Mitf* mRNA expressions. Argan oil treatment causes MITF phosphorylation which subsequently inhibited the transcription of melanogenic enzymes, TYR and DCT. The inhibitory effect of argan oil on melanin biosynthesis may be attributed to tocopherols as well as the synergistic effect of its components. The results of this study provide the scientific basis for the traditionally established benefits of argan oil and present its therapeutic potential against hyperpigmentation disorders.

1. Introduction

The use of argan oil to moisturize the skin and to maintain a fair complexion has been an established tradition among Moroccan women. *Argania spinosa* (L.) Skeels (Sapotaceae) is a tree species endemic to Morocco and has a great ecological and socioeconomic value in this area. The fruit of *A. spinosa* has an oleaginous kernel from which a well-known oil, "argan oil," is used in folk medicine and in cosmetics [1], especially in the southwestern region [2]. Traditionally, cosmetic argan oil was used to cure all kinds of skin pimples as well as juvenile acne and chicken pox pustules scars. It is also recommended to reduce dry skin matters and slow down the appearance of wrinkles. This oil is also used to treat psoriasis, eczema, joint pain, skin inflammation, and scabies, to heal burns and wounds, to cure brittle fingernails, to prevent hair loss and dry hair [3]. Argan oil for cosmetic use is cold-pressed oil extracted from unroasted kernels of argan fruit, which at present has gained worldwide recognition. It has been referred to as the "most expensive vegetable and cosmetic oil" [4] and as such has been a source of income for many Moroccan women who are members of cooperatives run by women. In the southern part of Morocco, cooperatives produce argan oil for food or for cosmetic use. The extraction of argan oil for food and cosmetic use is still being done using the traditional method. Argan oil is rich in tocopherols, medium chain fatty acids, carotenoids [5–7], squalene, and oleic acid [8].

Acquired hyperpigmentation after exposure to sunlight for a long time or such as in melasma and postinflammatory melanoderma is characterized by an increase in production and accumulation of melanin [9]. The biosynthesis of melanin in the pigment cells is catalyzed by the melanogenic enzymes tyrosinase, tyrosinase-related protein 1, and dopachrome tautomerase [10, 11]. Hyperpigmentation occurs when there is an increase in the activity of these enzymes due to triggering factors such as ultraviolet light or chronic inflammation [12, 13]. Different approaches to address this problem have been the subject of most researches [14–17]. Depigmenting agents are applied not just for the prevention and treatment of irregular hyperpigmentation in Western countries, but to make the skin whiter based on traditional beliefs in Asia [18].

The use of chemical agents such as hydroquinone and kojic acid, among many others, is the most popular way of managing hyperpigmentation [9, 19, 20]. However, undesirable side effects such as allergy [21], exogenous ochronosis, infectious dermatosis, and, in particular, extended dermatophytosis and necrotizing cellulitis, contact eczema, and certain hyperpigmentation when depigmenting agents were discontinued [22]. It is for these unwanted effects that the use of naturally occurring and locally available depigmenting agents still seems to be the most practical way to manage hyperpigmentation [23].

The depigmenting effect of the components of argan oil such as free fatty acids, tocopherols [24, 25], and the ability of carotenoids to protect the skin from sunlight [26] has been reported. However, there has been no study on the effect of whole argan oil on the melanin biosynthesis using a murine cell culture model. This study presents the use of argan oil as a depigmenting agent and the elucidation of the mechanism underlying its effects is also presented.

2. Materials and Methods

2.1. Origin of Argan Oil Sample and Oil Components Composition Analysis. Argan oil used in the experiment was kindly provided by Alain-Claude Kerrien (Naturelle d'argan, Marrakech, Morocco). It was cosmetic-type argan oil obtained, in a women argan cooperative (Cooperative Agdal, Essaouira Region, Morocco), by mechanical press of unroasted almonds of argan tree (*Argania spinosa L. Skeels,* Sapotaceae).

Its fatty acid and minor components composition were determined according to standard procedures: NF EN ISO 5508 for fatty acids composition (methyl esters in %), ISO 9936 for the composition of tocopherols, and NF ISO 6799 for the composition of sterol fraction.

2.2. Antioxidant Activity of Argan Oil

2.2.1. β-Carotene/Linoleic Acid Bleaching Assay. The β-carotene/linoleic acid bleaching assay was performed following the method described by Miraliakbari and Shahidi [27] with slight modification. A mixture of β-carotene and linoleic acid was prepared by dissolving 0.5 mg β-carotene in 1 mL chloroform (HPLC grade) and 25 μL linoleic acid in 200 mg Tween 20. The chloroform was then completely evaporated under vacuum, and 100 mL of distilled water was subsequently added to the residue, and then the mixture was shaken vigorously to form an emulsion. From this emulsion, 2.5 mL was transferred into different test tubes containing 350 μL of samples in acetone at different concentrations. All samples were vortexed for 1 min and placed at 50°C in a water bath for 2 h together with a negative control (blank) which contained the same volume of acetone instead of the samples. The absorbance of samples was measured at 470 nm using a spectrophotometer at initial time ($t = 0$) against a blank (emulsion without β-carotene). A standard BHT was used as a control.

Antioxidant activities (inhibitions percentage, I%) of the samples were calculated using the following equation:

$$\mathrm{I\%} = \left(\frac{\mathrm{A}_{\beta\text{-carotene after 2 h assay}}}{\mathrm{A}_{\text{initial of } \beta\text{-carotene} \times 100}} \right) \times 100, \quad (1)$$

where $\mathrm{A}_{\beta\text{-carotene after 2 h assay}}$ is the absorbance values of β-carotene after 2 h assay remaining in the samples and $\mathrm{A}_{\text{initial of } \beta\text{-carotene}}$ is the absorbance value of β-carotene at the beginning of the experiments.

All tests were carried out in triplicate, and inhibition percentages were reported as means SD of triplicates.

2.2.2. Reducing Power Activity. The reductive potential of argan oil was determined following the method of Bounatirou et al. [28]. Different concentrations of argan oil in acetone (1 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, and pH 6.6) and potassium ferricyanide (2.5 mL, 1% w/v). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%, 2.5 mL) was added to the mixture and centrifuged for 10 min at 1200 g. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured using a spectrophotometer (700 nm). The extract concentration providing 0.5 of absorbance (IC50) was calculated by plotting the absorbance at 700 nm against the corresponding argan concentration. BHT and ascorbic acid were used as reference compounds.

2.3. Cell Culture. The B16 murine melanoma cells (B16 cells) used in the experiment were purchased from the Riken Cell Bank in Tsukuba, Japan, and maintained as a monolayer culture in Dulbecco's Modified Eagle's Medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), 4 mm l-glutamine (Sigma), 50 units/mL penicillin, and 50 μg/mL streptomycin (Cambrex, East Rutherford, NJ, USA) at 37°C in a humidified atmosphere of 5% CO_2.

2.4. Melanin Quantification. The melanin content synthesized by B16 melanoma cells treated with argan oil was determined as previously described [16]. B16 cells were seeded onto 100 mm petri dishes at a density of 5×10^5 cells/dish and incubated at 37°C in a 5% CO_2 atmosphere. After overnight incubation, the growth medium was replaced with a fresh

medium containing arbutin (100 μM) or argan oil (1/100, 1/1,000, or 10,000 v/v) dilutions. After 48 h incubation, the growth medium was then removed, and then the cells washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization (0.25% trypsin/0.02% EDTA in PBS; Sigma). The cells were pelleted and 0.1% Triton X-100 was added to solubilize the cell membrane. The synthesized melanin was then purified and precipitated in 10% trichloroacetate, and the melanin dissolved in 1 mL 8 N NaOH for 2 h at 80°C. The absorbance of the melanin solution was measured at 410 nm, and the melanin content was calculated using a standard curve for synthetic melanin. The cell viability and total cell count were assessed using the ViaCount Program of Guava PCA (GE Healthcare, UK Ltd., Buckinghamshire, UK) following the manufacturer's instructions. The melanin content was expressed as melanin content/cell (% of control).

2.5. Western Blot. The protein samples used for western blotting were extracted from B16 cells seeded onto 100 mm dishes at a density of 3×10^6 cells per dish and cultivated as described above. After the cells were allowed to attach overnight, the medium was replaced with medium containing hirsein A (HA), a melanogenesis inhibitor [17], or argan oil (1/10,000, 1/1,000, or 1/100) and incubated for additional 24 or 48 h. The medium was then removed, and the cells washed twice with PBS before the total protein was extracted using RIPA buffer (Sigma, USA) according to the manufacturer's instructions. Protein sample (15 μg) was resolved in 10% SDS-polyacrylamide gel by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blotted with primary antibodies for tyrosinase, (MITF) microphthalmia-associated transcription factor, tyrosinase (TYR), tyrosinase-related protein 1 (TRP1), dopachrome tautomerase (DCT), p-ERK1/2 (Sigma), ERK2 (Sigma), and GAPDH (Santa Cruz Biotechnology, Inc., USA). The signal was visualized using LiCor Odyssey Infrared Imaging System after reaction with goat anti-mouse IRDye 680LT, donkey anti-goat IRDye 800CW (LI-COR), or goat anti-rabbit IRDye 800CW (LI-COR).

2.6. Quantitative Real-Time PCR Analysis. B16 cells were seeded onto 100 mm dishes at a density of 3×10^6 cells per dish and cultivated as described above. After the cells were allowed to attach overnight, the medium was then replaced with medium with or without argan oil (1/10,000, 1/1,000, or 1/100) and incubated for additional 24 h. The medium was then removed, and the cells washed twice with PBS before the total RNA was extracted using ISOGEN kit (Nippon Gene, Tokyo, Japan) and quantified using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies). The RNAs were then used as templates for reverse transcription polymerase chain reaction (RT-PCR) using the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. To quantify the mRNA expression of the tyrosinase (*Tyr*) gene, quantitative real-time polymerase chain reaction (RT-PCR) analysis was performed with a 7500 Fast Real-Time PCR system using TaqMan Universal PCR mix and TaqMan probes (Applied Biosystems, Foster City, CA, USA) with the following thermal cycling protocol: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. *Gapdh* was used as a control. Specific TaqMan primers for *Tyr, Trp1, Dct, Mitf*, and *Gapdh* were obtained from Applied Biosystems (Foster City, CA, USA). Data were analyzed using 7500 Fast System SDS Software 1.3.1. (Applied Biosystems).

Table 1: Major fatty acids content of the argan oil used in the experiment.

Fatty acid	Amount (%)
Palmitic acid	12.2
Stearic acid	6.23
Oleic acid	48.5
Linoleic acid	28.47

Table 2: Tocopherols present in the argan oil used in the experiment[a].

Tocopherols	(mg/100 g argan oil)
Total tocopherols	63.5
Alpha-tocopherol	44
Beta-tocopherol	10
Delta-tocopherol	8
Gamma-tocopherol	1

[a]Analysis performed by the Etablissement Autonome de Controle et de Coordination des Exportations (EACC) "Agadir, Morocco."

2.7. Statistical Analysis. Results were expressed as mean value ± SD, and the differences between treated and control treatments were evaluated for significance using Student's *t*-test. Differences between the means at the 5% confidence level were considered significant.

3. Results

3.1. Chemical Analyses. The chemical analyses of the cosmetic argan oil used in the experiments were performed. Table 1 shows the amount of the major fatty acid in argan oil oleic acid (46.1%), linoleic acid (34.5%), palmitic (12.2%), and stearic acid (6.23%). The amount of tocopherols present in the sample was also evaluated and results showed that the total tocopherol was 63.5 mg/100 g oil with alpha-tocopherol as the main tocopherol (44 mg/100 g oil) (Table 2). The chemical analysis also revealed the presence of triterpene alcohols, carotenoids and xanthophylls. The sterols present in argan oil are β-sitosterol (highest concentration), spinasterol, schottenol, and β-sitosterol. Other components include carotenoids (2 mg/kg) and xanthophylls (1.7 mg/kg) (data not shown).

3.2. Antioxidant Activity and Reductive Potential of Argan Oil. The antioxidant activity of argan oil was determined using the β-carotene–linoleate model system and compared with that of BHT. The results showed that argan oil prevented the bleaching of β-carotene and therefore may contribute to

Table 3: Antioxidant activity of argan oil and synthetic antioxidant (BHT and ascorbic acid) in β-carotene bleaching assay and reducing power methods.

Sample	β-carotene (EC_{50}, μg/mL)	Reducing power (EC_{50}, μg/mL)
Argan oil	93.02 ± 5.74	800.31 ± 0.8
BHT	50.31 ± 0.07	31.33 ± 0.03
Ascorbic acid		6.89 ± 0.01

the lipoperoxidation protection (Table 3). Furthermore, to determine argan oil's ability to act as an electron donor, its reductive potential was also evaluated. The electron donor reacts with free radicals, converts them to more stable products, and finally terminates radical chain reactions. The reductive capacity of a compound is recognized as a significant indicator of its potential antioxidant activity [28]. Argan oil showed some degree of electron-donating capacity, but it was still lower than that of synthetic antioxidant BHT (EC50 = 31.33 ± 0.03 μg/mL) and ascorbic acid (EC_{50} = 6.89 ± 0.01 μg/mL).

3.3. Effect on the Melanin Content in B16 Cells. The amount of intracellular melanin in B16 cells treated with argan oil was quantified. Argan oil was mixed with the growth medium and sonicated prior to treatment. Cells treated with 1/100, 1/1000, and 1/10000 v/v of argan oil were then incubated for 48 h and the melanin content was quantified as described in the Materials and Methods Section. Results showed that treatment with argan oil caused a dose-dependent decrease in the melanin content of B16 cells. Cell viability assay results revealed that except for 1/100 dilution, argan oil does not have any cytotoxic effect on B16 cells. Longer incubation time (72 hours) revealed a time-dependent significant decrease in the melanin content of the cells (Figure 1). Compared to arbutin, a known melanogenesis inhibitor, cells treated with argan oil for 72 h had almost the same amount of melanin as the arbutin-treated cells. The color of the cell lysates dissolved in NaOH clearly shows the depigmenting effect of argan oil (Figure 1(b)).

3.4. Melanogenic Enzymes Expressions in B16 Cells. Melanin biosynthesis is catalyzed by three major enzymes: tyrosinase (TYR), tyrosinase-related protein 1 (TRP1), and dopachrome tautomerase (DCT). Here, the expressions of these enzymes were determined by western blotting using specific antibodies, and the results showed that treatment with 1/1000 v/v argan oil inhibited the expression of tyrosinase and Dct proteins in a time-dependent manner, the significant inhibition of which was at after 72 hrs of treatment (Figure 2(a)). Like tyrosinase, there was a significant decrease in DCT expression which was not observed in TRP1. Densitometric analysis of the protein expressions showed that there was about 60% decrease in TYR expression and 80% in DCT protein levels (Figure 2(b)). It was, however, noted that the expression of TRP1 was not significantly decreased with only about 10% decrease in its expression after 72 h of treatment with argan oil (compared to the untreated control). As expected, the positive control hirsein A significantly inhibited the expressions of all the three enzymes [17].

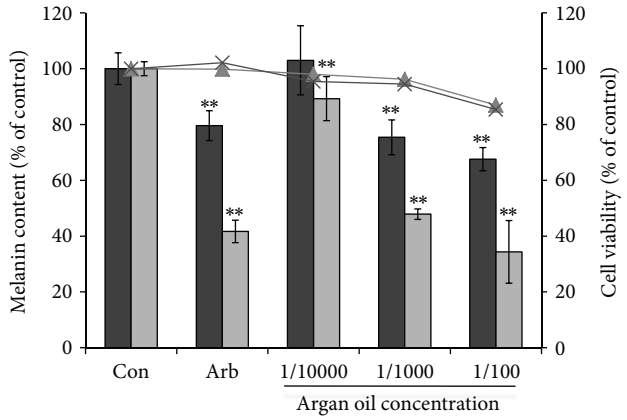
3.5. Molecular Mechanism Underlying the Depigmenting Effect. The expressions of the melanogenic enzymes' genes are regulated by the MITF that binds on the regulatory element of *Tyr*, *Trp1*, and *Dct* genes. The expressions of total and phosphorylated ERK1/2 and MITF were evaluated using western blot, the results of which revealed a significant increase in the activation of ERK 24 h after argan oil treatment (Figure 3(a)). Compared to hirsein A which had significant ERK phosphorylation 1 h after treatment, argan oil appears to induce this phosphorylation after 24 h. Likewise, an increase in the level of phosphorylated MITF expression (Figure 3(a)) and the ratio of the phosphorylated to the total MITF was observed 48 h after treatment with argan oil (Figure 3(b)).

To confirm if the increase in the phosphorylation of MITF led to a decrease in the expressions of the *Tyr*, *Trp1*, or *Dct* mRNA, real-time PCR was performed. Results showed that argan oil significantly downregulated the expressions of *Tyr* and *Dct* mRNA but not *Trp1* (Figure 4(a)). To determine if argan oil has an effect on the expression of MITF at the transcriptional level, the expression of *Mitf* gene was quantified using real-time PCR. Results showed a downregulation of the *Mitf* mRNA expression (Figure 4(b)).

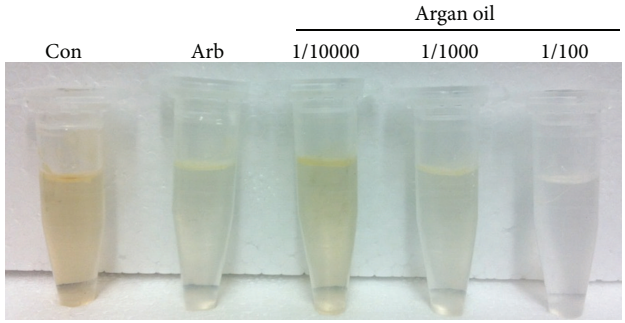
4. Discussion

The health benefits of argan oil consumption have been widely reported. Argan oil confers cancer chemopreventive [8] and hypolipidemic effects [29], among others. Here, the effect of argan oil on the skin was tested *in vitro* using the model cell line B16 murine melanoma cells. Argan oil has been used by Moroccan women as cosmetic oil for centuries, but it is only until recently that the demand for argan oil has spread beyond North Africa. European chemists recently discovered that argan oil exhibits desirable biochemical properties for cosmetics sold commercially throughout Europe, Israel, and around the world [30].

Skin pigmentation depends on the amount of melanin and its deposition on the skin. During melanin production, pigment cells, such as B16 cells, depend on the amount of tyrosinase in the cells or on compounds/factors that inhibit or activate this enzyme [31–33]. Arbutin, kojic acid, and ascorbic acid are just a few of known compounds that can inhibit melanin biosynthesis [34]. The effectivity of depigmenting agents on melanogenesis is evaluated by quantifying the melanin content of the pigment cells. The biosynthesis of melanin occurs in the melanosomes that contain the enzymes tyrosinase (TYR), tyrosinase-related protein 1 (TRP1), and dopachrome tautomerase (DCT). The first two rate-limiting reactions of melanogenesis are catalyzed by tyrosinase and these are (1) the hydroxylation of tyrosine giving the 3, 4-dihydroxyphenylalanine (DOPA) and (2) the oxidation of dopa to DOPAquinone. In mice, Trp1 has been shown to oxidize 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) to indole-5, 6-quinone-2-carboxylic acid, which is not observed



(a)

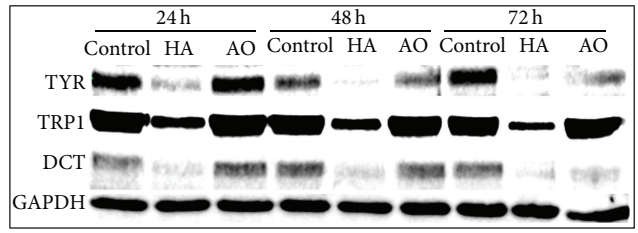


(b)

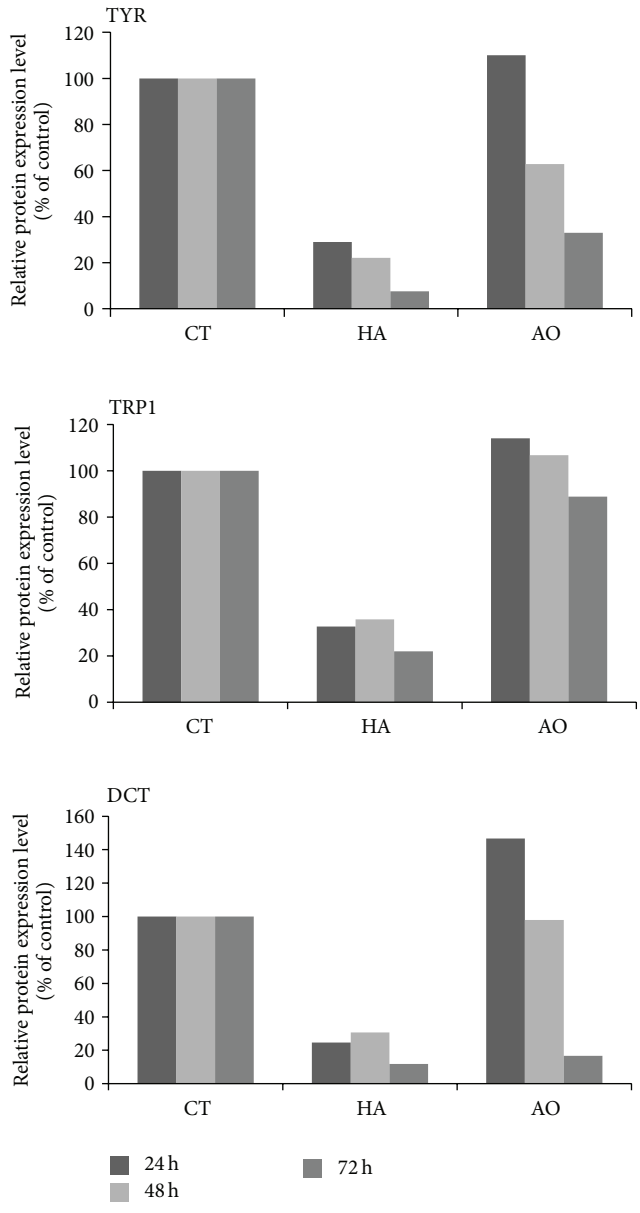
Figure 1: Effect of argan oil (AO) at 1/10000, 1/1000, and 1/100 (v/v) on the (a) melanin content (bar graph) and cell viability (line graph) of B16 melanoma cells cultured in 100 mm dish at a density of 5×10^5 cells/dish. (b) Lysates of cells treated with or without arbutin (100 mM) or argan oil. B16 cells were treated with or without arbutin or argan oil serially diluted in Dulbecco's Modified Eagles Medium and incubated for 48 or 72 h prior to melanin assay. *Statistically significant ($P < 0.05$) difference between treated and untreated (Con) cells.

in human TRP1. DCT isomerizes dopachrome to DHICA [34].

In this study, we evaluated the effect of argan oil on melanogenesis to validate the traditional belief that it can maintain a fair complexion. Argan oil, mixed with the growth medium of B16 cells incubated for 48 to 72 hours, decreased the melanin content of the cells (Figures 1(a) and 1(b)). The observed depigmentation was found to be due to the decrease in the levels of the melanogenic enzymes, with a significant decrease in TYR and DCT than in TRP1 (Figure 2). Tyrosinase is the rate-limiting enzyme of melanogenesis and is transported to the melanosomes where it mediates the first two steps of melanin synthesis. The decrease in the melanin content was observed after 48 h of treatment, but the most significant inhibitory effect was observed after 72 h. Although 1/10000 dilution can already cause a depigmenting effect,



(a)



(b)

Figure 2: Effect of argan oil on the expressions of the tyrosinase (TYR), tyrosinase-related protein 1 (TRP1), and dopachrome tautomerase (DCT) proteins determined by western blotting (a) and their densitometric values (b). B16 melanoma cells were cultured in 100 mm dish at a density of 3×10^6 cells/dish and treated without (Con or CT) or with 1 μM hirsein A (HA) or 1/1000 v/v argan oil (AO).

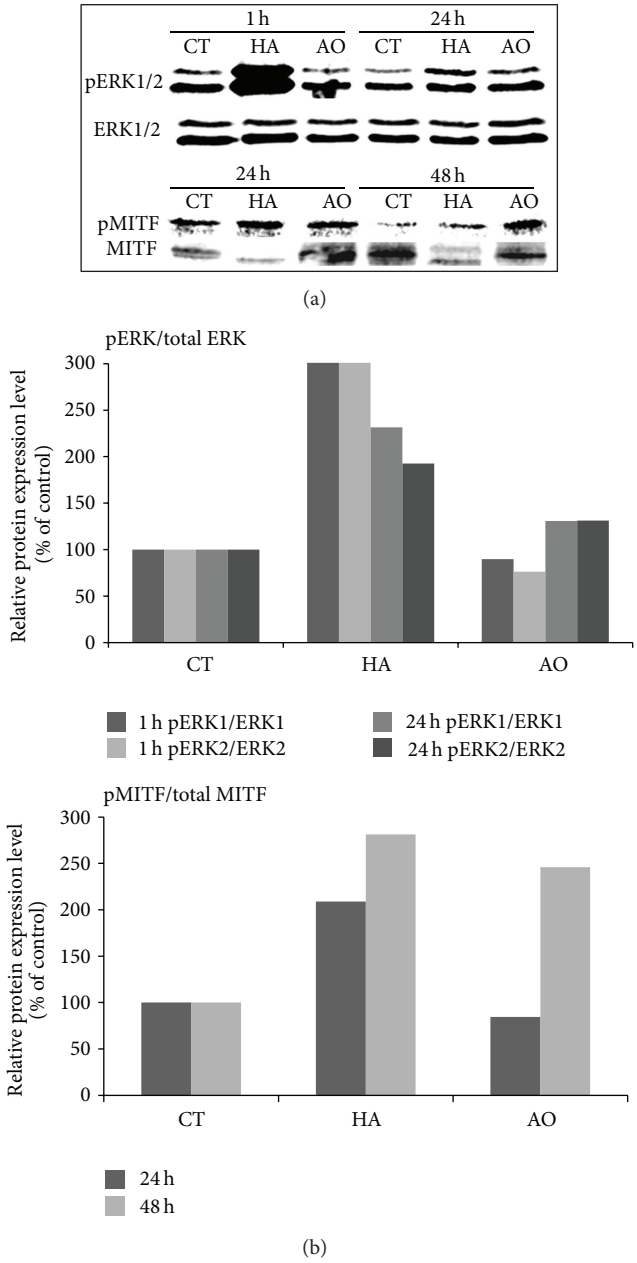


Figure 3: Effect of argan oil on the expressions of the phosphorylated extracellular signal-regulated kinases 1/2 (pERK1/2), ERK1, ERK2, phosphorylated microphthalmia-associated transcription factor (pMITF), and total MITF proteins (a) and their densitometric values (b). B16 melanoma cells were cultured in 100 mm dish at a density of 3×10^6 cells/dish and treated without (CT) or with 1 μM hirsein A (HA) or 1/1000 v/v argan oil (AO).

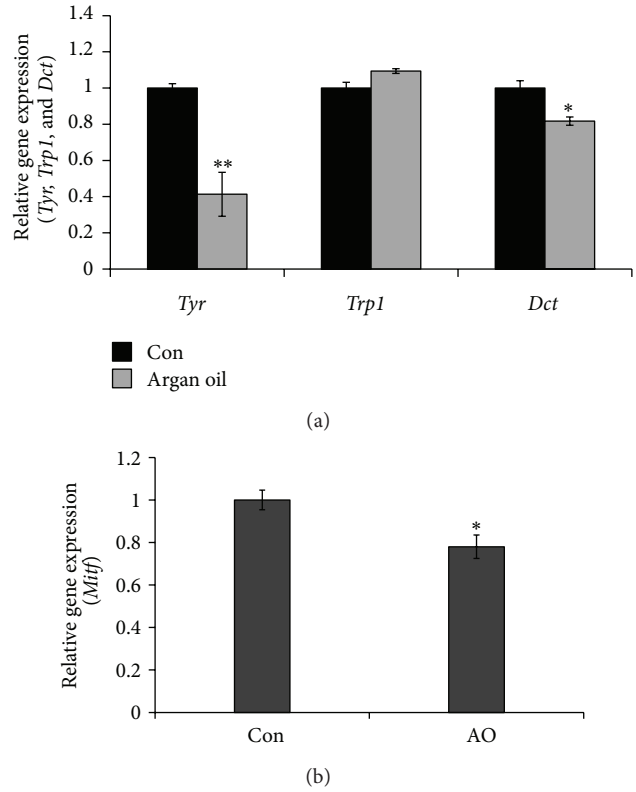


Figure 4: Effect of argan oil on the (a) tyrosinase (*Tyr*), tyrosinase-related protein 1 (*Trp1*), and dopachrome (*Dct*) and (b) microphthalmia-associated transcription factor (*Mitf*) mRNA expressions quantified using TaqMan real-time PCR. B16 melanoma cells were cultured in 100 mm dish at a density of 3×10^6 cells/dish and treated without (Con) or with 1/1000 v/v argan oil and incubated for 24 h prior to total RNA extraction.

significant melanin synthesis inhibitory effect without cytotoxicity was observed in 1/1000 dilution, even when compared to 100 μM arbutin, a known melanogenesis inhibitor [35]. The effect of argan oil at 1/100 dilution was also significant but also caused a slight decrease in the cell count and cell viability. We then determined the expression of tyrosinase as affected by argan oil treatment by western blot. Results show that the expression of the tyrosinase enzyme was decreased when cells were treated with argan oil (Figure 2). Although an immediate decrease in the concentration, such as after 4 h of treatment (Figure 2(a)), was not observed prolonged incubation with argan oil caused a significant decrease in the enzymes expressions (Figure 2(b)). Argan oil appears to not have any inhibitory effect on TRP1 and this is not surprising because although TRP1, and DCT share ~40% amino acid homology with TYR [36, 37], *Trp1* has a structure and promoter sequence very different from *Tyr* [38]. However, the reason for the observed effect of argan oil on TRP1 expression remains unclear.

In order to understand the underlying mechanism for the decrease in protein levels of the melanogenic enzymes, their expressions at the transcription level were also determined and the results show that their mRNA levels were also decreased by argan oil treatment (Figures 3(a) and 3(b)). The control of the expression of *Tyr*, the rate-limiting melanogenesis, depends in part on the regulation at the transcriptional level [39]. Some melanogenesis inhibitors such as TPA have been found to decrease the *Tyr* mRNA levels in treated cells [40], and in the case of argan oil, the same phenomenon was observed. As shown in Figure 4(a), the level of expression of the *Tyr* mRNA was decreased following argan oil treatment.

Melanogenic enzymes' gene expressions are regulated by the MITF, and our results showed an increase in the activation of MITF. The phosphorylation of MITF is linked to its subsequent degradation [41]. MITF plays an important role in melanocyte differentiation, proliferation, and survival [42]. To determine the probable cause of the increase in the activation of MITF, a time-dependent increase in the phosphorylation of ERK was performed the results of which show an increase in ERK1/2 activation by argan oil treatment (Figure 3). Compared to hirsein A, observed to have increased phosphorylation after one hour of treatment, argan oil appears to significantly increase ERK phosphorylation after 24 h of treatment. It has been established that sustained activation of the MAPK ERK leads to MITF degradation [41, 43]. Upon activation, the ERKs either phosphorylate a number of cytoplasmic targets or migrate to the nucleus where they phosphorylate and activate a number of transcription factors such as MITF. MAP kinase, ERK2, phosphorylates MITF, thereby targeting the transcription factor to proteasomes for degradation. Thus, in addition to the complex transcriptional regulation, melanogenesis is also subjected to a posttranslational regulation that controls MITF or TYR function [44].

As summarized in Tables 1, 2, and 3, argan oil is rich in tocopherols and unsaturated fatty acids such as oleic acid and linoleic acid. Argan oil shows an interesting antioxidant activity in β-carotene/linoleic acid bleaching assay, predicting a lipoperoxidation protection. The skin whitening effect of alpha-tocopherol or commonly known as vitamin E has been reported [45]. Oral intake of vitamin E can improve facial hyperpigmentation, especially in combination with vitamin C. The exposure to UV radiation induces lipid peroxidation in the skin, which can cause damage to the epidermal cells, leading to postinflammatory hyperpigmentation. This depigmenting effect of alpha-tocopherol is attributed to its antioxidative effects [46] and tyrosinase activity inhibitory effects [47].

The antioxidant and reducing properties of argan oil provide additional benefit to the cells via the reduction of oxidative stress in the absence of melanin. Melanin has a free radical scavenging property in pigment cells. In conditions where melanin is decreased, such as in cells treated with argan oil or in melanocytes deprived of melanin such as in OCA2 cells, oxidative stress inhibitors may provide protection from oxidative damage [48, 49]. Fujiwara et al. [48] reported that vitamin E or tocopherol simultaneously administered with vitamin C traps the peroxyradical caused by UV-B, acts as a radical scavengers and inhibit, the production of melanogenic cytokines, and this appears to be due to the synergistic effect of vitamins A and C on B16 melanoma cells. This mechanism could also explain how, in this study, the different components of argan oil worked collectively to inhibit melanin synthesis in B16 cells. Argan oil, as well as its components, is known to have antioxidant and antiaging properties and can therefore alleviate oxidative stress in the cells, but this effect could be independent of its effect on melanogenesis. Alpha-MSH, for example, a known melanogenesis stimulator, has an oxidative stress inhibitory effect on human melanocytes and even on cells that have lost their ability to produce melanin (cells with *OCA2* mutation) providing evidence that its antioxidant effect may not be directly related to its melanogenesis regulatory effects [49]. The antioxidant effect of argan oil may serve to protect the cells from oxidative damage from UV radiation in the absence of melanin following its depigmenting effect on the skin, thus giving a scientific basis to the traditionally claimed effect on the reduction of the rate of appearance of wrinkles and in fighting dry skin [3].

Although tocopherols appear to be the main component that influences the overall effect of argan oil on melanogenesis, the effect of other components may also play an important role. Argan oil also contains free fatty acids with reported effects on tyrosinase activity. Linoleic acid has been reported to decrease tyrosinase activity, while palmitic acid increases it [40]. The argan oil sample used in this study has 28.47% linoleic acid, and 12.2% palmitic acid and although it can be induced that the high linoleic content can cause a decrease in the tyrosinase activity, the results of this study showed that argan oil does not affect mushroom tyrosinase activity (data not shown). Obviously, and as reported by Ando et al. [40], tyrosinase activity is not correlated with the *Tyr* mRNA level. The overall skin depigmenting effect due to the inhibition of melanogenic enzymes (TYR and DCT) expressions can therefore be attributed to the main components (tocopherols) and/or the synergistic effect of all the argan oil components.

5. Conclusion

There is a renewed interest in plants as pharmaceuticals in the Western world and this is on the discovery of new biologically active molecules and into the adoption of crude extracts of plants for self-medication by the general public [47]. North African traditional medicine, therefore, has an important contribution in the maintenance of health in all parts of the world and in the introduction of new treatments. And although some may view the isolation of compounds and their use as single chemical entities as a better option and has resulted to the replacement of plant extracts use, nowadays, a view that there may be some advantages to the medical use of extracts as opposed to isolated single compounds, is gaining popularity [50, 51]. Published reports on the therapeutic benefits of consumption of argan oil have been summarized by [52], but its effect on pigment cells has not yet been reported. Here, we present argan oil as an effective melanin biosynthesis inhibitor, the effect of which may be contributed by the individual components or their synergistic effect. Although the effect of argan oil components (tocopherols, fatty acids, etc.) on melanin biosynthesis has been reported, this is the first report on the inhibitory effect of argan oil on melanogenesis in B16 cells, providing the scientific basis for the centuries-long knowledge of its beneficial effect on the skin.

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Research Article

HSP90 Inhibitors, Geldanamycin and Radicicol, Enhance Fisetin-Induced Cytotoxicity via Induction of Apoptosis in Human Colonic Cancer Cells

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We revealed the cytotoxic effect of the flavonoid, fisetin (FIS), on human COLO205 colon cancer cells in the presence and absence of the HSP90 inhibitors, geldanamycin (GA) and radicicol (RAD). Compared to FIS treatment alone of COLO205 cells, GA and RAD significantly enhanced FIS-induced cytotoxicity, increased expression of cleaved caspase-3 and the PAPR protein, and produced a greater density of DNA ladder formation. GA and RAD also reduced the MMPs with induction of caspase-9 protein cleavage in FIS-treated COLO205 cells. Increased caspase-3 and -9 activities were detected in COLO205 cells treated with FIS+GA or FIS+RAD, and the intensity of DNA ladder formation induced by FIS+GA was reduced by adding the caspase-3 inhibitor, DEVD-FMK. A decrease in Bcl-2 but not Bcl-XL or Bax protein by FIS+GA or FIS+RAD was identified in COLO205 cells by Western blotting. A reduction in p53 protein with increased ubiquitin-tagged proteins was observed in COLO205 cells treated with FIS+GA or FIS+RAD. Furthermore, GA and RAD reduced the stability of the p53 protein in COLO205 cells under FIS stimulation. The evidence supports HSP90 inhibitors possibly sensitizing human colon cancer cells to FIS-induced apoptosis, and treating colon cancer by combining HSP90 inhibitors with FIS deserves further in vivo study.

1. Introduction

Colorectal cancer is one of the leading causes of cancer deaths in western countries and has become a common malignancy in Asia due to significant changes in diet and lifestyle over the past few decades. A combination of surgery, chemotherapy, and targeted therapy is the mainstream anticancer therapy; however, there are possible systemic toxic effects with such chemical compounds. In contrast, flavonoids are natural dietary compounds. Epidemiologic studies showed that high intake of flavonoid-enriched vegetables and fruits can reduce the risk of colon cancer [1]. In vitro studies revealed that flavonoids can reduce the risk of colon cancer via their chemopreventive properties including induction of cell-cycle arrest and apoptosis and their antiproliferative effect, thus providing a novel therapeutic option for cancer treatment by complementary and alternative medicine [2].

Fisetin (3,7,3,4-tetrahydroxyflavone; FIS) is one of the major flavonoids widely found in fruits and vegetables including apples, onions, grapes, and cucumbers and was shown to exert a variety of biological activities, including antioxidant, anti-inflammatory, anti-invasive, and antiproliferative effects. FIS was reported to inhibit the proliferation of several cancer cells, including hepatocellular carcinoma, prostate cancer, and colon cancer cells. Additionally, FIS may also act as an inhibitor of cyclin-dependent kinases (CDKs) to induce cell-cycle arrest in cancer cells [3]. Furthermore, FIS was reported

to induce apoptosis in different cancer cells, including hepatocellular carcinoma SK-HEP-1, myeloleukemic HL-60, and prostate cancer LNCaP cells [4–6]. Recent studies showed that FIS was able to induce apoptosis in both p53-wild-type and p53-mutant colon cancer cells [7, 8]. These accumulating results provide evidence of the potent anticancer activity of FIS; however, the effects of FIS on apoptosis of colon cancer cells and its underlying mechanisms have yet to be clearly elucidated.

Heat shock protein 90 (HSP90) is an essential chaperon for integrity and function of a wide range of oncogenic client proteins which are implicated in carcinogenesis [9]. Compared to normal tissues, HSP90 is generally overexpressed in tumors by around 2~10-fold and is associated with a poor prognosis [10–12]. Through its ability to control the stability and activity of client proteins involved in the oncogenic process, targeting HSP90 has the potential to affect most of the hallmarks of cancer. Since the first HSP90 inhibitor, geldanamycin (GA), was found in 1994, HSP90 inhibitors have emerged as a promising therapeutic intervention for a wide variety of human cancers in the past two decades [13]. Nowadays, colorectal cancer expressing epidermal growth factor receptor (EGFR), a client protein of HSP90, has been targeted using tyrosine kinase inhibitors and monoclonal antibodies [14].

In this study, we investigated the role of HSP90 inhibitors in the anticancer effects of FIS against human colon cancer cells. The HSP90 inhibitors, geldanamycin (GA) and radicicol (RAD), effectively enhanced the cytotoxicity of human COLO205 colon cancer cells under FIS stimulation. GA and RAD induced apoptosis of FIS-treated COLO205 cells through disruption of the mitochondrial membrane potential (MMP) and decreased Bcl-2 and p53 protein expressions via enhancement of protein ubiquitination and reduction of p53 protein stability. Our results provide a molecular basis for treating human colon cancer with FIS and HSP90 inhibitors.

2. Materials and Methods

2.1. Cell Culture. COLO205 colonic carcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI 1640 supplemented with antibiotics (100 U/mL penicillin A and 100 U/mL streptomycin) and 10% heat-inactivated fetal bovine serum (FBS; Gibco/BRL, Grand Island, NY, USA) and maintained in a 37°C humidified incubator containing 5% CO_2.

2.2. Agents. The chemical reagents of FIS, GA, RAD, BCIP, MTT, and nitroblue tetrazolium (NBT) were obtained from Sigma Chemical (St. Louis, MO, USA). Antibodies of α-tubulin, poly(ADP-ribose)polymerase (PARP), caspase-3, caspase-9, Bcl-2, and Bax were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The colorigenic synthetic peptide substrates, Ac-DEVD-pNA (caspase-3 substrate) and Ac-YVAD-pNA (caspase-9 substrate), and the protease inhibitor for caspase-3 (Ac-DEVD-FMK) were purchased from Calbiochem (Darmstadt, Germany).

2.3. Cell Viability Assay. Cell viability was assessed by MTT staining. Briefly, cells were plated at a density of 10^5 cells/well into 24-well plates. At the end of treatment, the supernatant was removed, and 30 μL of the tetrazolium compound, MTT, and 270 mL of fresh RPMI medium were added. After incubation for 4 h at 37°C, 200 μL of 0.1 N HCl in 2-propanol was placed in each well to dissolve the tetrazolium crystals. Finally, the absorbance at a wavelength of 600 nm was recorded using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, MA, USA).

2.4. Western Blotting. Total cellular extracts (30 μg) were prepared and separated on 8% sodium dodecylsulfate (SDS)-polyacrylamide minigels for PARP detection and 12% SDS-polyacrylamide minigels for caspase-3, caspase-9, the Bcl-2 family, tERK, pERK, and α-tubulin detection and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated at 4°C with 1% bovine serum albumin and then incubated with the indicated antibodies for further 3 h at room temperature followed by incubation with an alkaline phosphatase-conjugated immunoglobulin G (IgG) antibody for 1 h. Protein was visualized by incubating with the colorimetric substrates, NBT and BCIP.

2.5. DNA Fragmentation Assay. Colonic carcinoma cells under different treatments were collected and then lysed in 100 μL of lysis buffer (50 mM Tris at pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium sarcosinate, and 1 mg/mL proteinase K) for 3 h at 56°C. Then, 0.5 mg/mL RNase A was added to each reaction for another 1 h at 56°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before loading. Then, DNA samples were mixed with 6 μL of loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) glycerol, and 0.025% (w/w) bromophenol blue) and loaded onto a 2% agarose gel containing 0.1 mg/mL ethidium bromide. The agarose gels were run at 100 V for 45 min in TBE buffer then observed and photographed under UV light.

2.6. Measurement of the MMP. After different treatments, cells were incubated with 40 nM DiOC6(3) for 15 min at 37°C then washed with ice-cold phosphate-buffered saline (PBS) and collected by centrifugation at 500 ×g for 10 min. Collected cells were resuspended in 500 μL of PBS containing 40 nM DiOC6(3). Fluorescence intensities of DiOC6(3) were analyzed on a flow cytometer (FACScan, Becton Dickinson) with respective excitation and emission settings of 484 and 500 nm.

2.7. Analysis of Caspase-3 and -9 Activities. Ac-DEVD-pNA was used as a colorimetric protease substrate to detect caspase-3 activity. After different treatments, cells were collected and washed three times with PBS and resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA. Cell lysates were clarified by centrifugation at 15,000 rpm for 3 min, and clear lysates containing 200 μg of protein were incubated with 100 mM of the indicated specific colorimetric

substrates at 37°C for 1 h. Alternative activities of caspase-3 and -9 enzymes were described by the cleavage of a colorimetric substrate and measuring the absorbance at 405 nm.

2.8. Statistical Analysis. Values are expressed as the mean ± standard error (SE) of triplicate experiments. The significance of the difference from the respective controls for each experiment was assayed using a one-way analysis of variance (ANOVA) for a post-hoc Bonferroni analysis when applicable, and P values of <0.05 or <0.01 were considered statistically significant.

3. Results

3.1. HSP90 Inhibitors, GA and RAD, Enhanced FIS-Induced Cytotoxicity via Inducing Apoptosis in COLO205 Cells. The chemical structures of FIS and its structurally related compound, robinetin (ROB), are depicted in Figure 1(a); ROB contains an additional OH group at C5 of FIS. Data of the MTT assay showed that FIS but not ROB at concentrations of 60 and 120 μM showed slight but significant reductions in the viability of COLO205 colon carcinoma cells by 11.7% ± 3.2% and 27.6% ± 4.7%, respectively (Figure 1(b)). Analysis of DNA integrity of COLO205 cells under FIS or ROB treatment showed that FIS at a concentration of 120 μM was able to reduce the integrity of DNA via increased DNA ladder formation in COLO205 cells according to DNA agarose electrophoresis (Figure 1(c)). In the presence of the HSP90 inhibitor, GA, an increase in DNA ladder intensity was observed in FIS-treated COLO205 cells (Figure 1(d)). Data of the MTT assay showed that the addition of GA significantly enhanced COLO205 cell death by FIS (Figure 1(e)). A significant increase in the ratio of hypodiploid cells (sub-G1) with GA was detected in FIS-treated COLO205 cells by a flow cytometric analysis (Figure 1(f)). Increased intensity of DNA ladders by another HSP90 inhibitor, RAD, was observed in COLO205 cells under FIS stimulation (Figure 1(g)). These data support that application of HSP90 inhibitors, such as GA and RAD, may potentiate the cytotoxic effect of FIS against the viability of COLO205 cells.

3.2. Induction of Caspase-3-Mediated Apoptosis in GA- or RAD-Enhanced FIS-Induced COLO205 Cell Apoptosis. We further investigated if activation of caspase-3 was involved in GA- or RAD-enhanced apoptosis of COLO205 cells under FIS stimulation. As shown in Figure 2(a), increases in cleaved caspase-3 and a caspase-3 substrate PARP protein were detected in GA- or RAD-treated COLO205 cells under FIS stimulation. No change in the expression of α-tubulin (α-TUB) was examined as an internal control to verify that similar amounts of protein were loaded in each lane. Additionally, GA at a concentration of 2 μM significantly increased caspase-3 and PARP cleavage in FIS-treated COLO205 cells (Figure 2(b)). FIS alone showed a slight but significant increase in caspase-3 activity, and a more significant increase in caspase-3 activity was detected in FIS+GA- or FIS+RAD-treated COLO205 cells using Ac-DEVD-pNA as a specific caspase-3 fluorescent substrate (Figure 2(c)). Data of DNA analysis showed that the addition of the peptidyl caspase-3 inhibitor, Ac-DEVD-FMK, inhibited FIS+GA-induced DNA ladder formation in COLO205 cells (Figure 2(d)). This indicated that GA- or RAD-enhanced FIS-induced cell death was mediated by activation of a caspase-3-mediated apoptotic pathway.

3.3. Loss of the MMP with Activation of Caspase-9 in GA- or RAD-Enhanced FIS-Induced COLO205 Cell Apoptosis. We further studied if GA- or RAD-enhanced apoptosis of FIS-treated COLO205 cells occurred through destroying mitochondrial homeostasis. The MMP was detected by DiOC6(3) staining, and results in Figure 3(a) show that FIS treatment did not affect the MMP, and a significant decrease in the MMP was detected in FIS+GA- and FIS+RAD-treated COLO205 cells, represented here as a decrease in the fluorescence intensity. GA and RAD alone showed no effect on the MMP in COLO205 cells. Additionally, induction of cleavage of the caspase-9 protein by FIS+GA or FIS+RAD was detected in COLO205 cells by Western blotting (Figure 3(b)). Measurement of caspase-9 activity using the specific fluorescent caspase-9 substrate, Ac-ZEVD-pNA, showed that the addition of GA or RAD induced caspase-9 activity in COLO205 cells under FIS stimulation; however, no change in caspase-9 activity was observed in FIS-, GA-, or RAD-treated COLO205 cells (Figure 3(c)). Bcl-2 family proteins were shown to regulate the MMP and play important roles in apoptosis. Among Bcl-2 family proteins, Bcl-2 and Bcl-XL are anti-apoptotic proteins, and Bax is a proapoptotic protein. In the presence of GA or RAD treatment, both Bcl-XL and Bax proteins remained unchanged in COLO205 cells under FIS stimulation; however, expression of the Bcl-2 protein was significantly reduced (Figure 4). Decreased Bcl-2 protein by RAD but not GA alone was observed, and similar results were obtained from three independent experiments. These data suggest that disruption of the MMP in accordance with induction of caspase-9 activation and a decrease in Bcl-2 protein expression was involved in GA and RAD enhancement of FIS-induced apoptosis of COLO205 cells.

3.4. GA Downregulates the p53 Protein at a Posttranscriptional Level in FIS-Treated COLO205 Cells. GA's induction of apoptosis that depends on regulation by p53 was previously reported; therefore, we investigated changes in the p53 protein with and without GA or RAD in FIS-treated COLO205 cells. As shown in Figure 5(a), a significant decrease in the p53 protein was detected in FIS+GA- or FIS+RAD-treated COLO205 cells, compared to that in control, FIS-, GA-, and RAD-treated cells. Additionally, increases in ubiquitin-targeted proteins were detected in FIS+GA- or FIS+RAD-treated COLO205 cells. This indicates that the reduction in the p53 protein by FIS+GA or FIS+RAD was probably due to protein degradation. To determine whether this was the case, we measured the half-life of the p53 protein by treating cells with FIS, GA, RAD, and cycloheximide (CHX), an inhibitor of protein synthesis. As shown in Figure 5(b), the p53 protein in COLO205 cells is a very stable protein that exhibits a long half-life, and the p53 protein

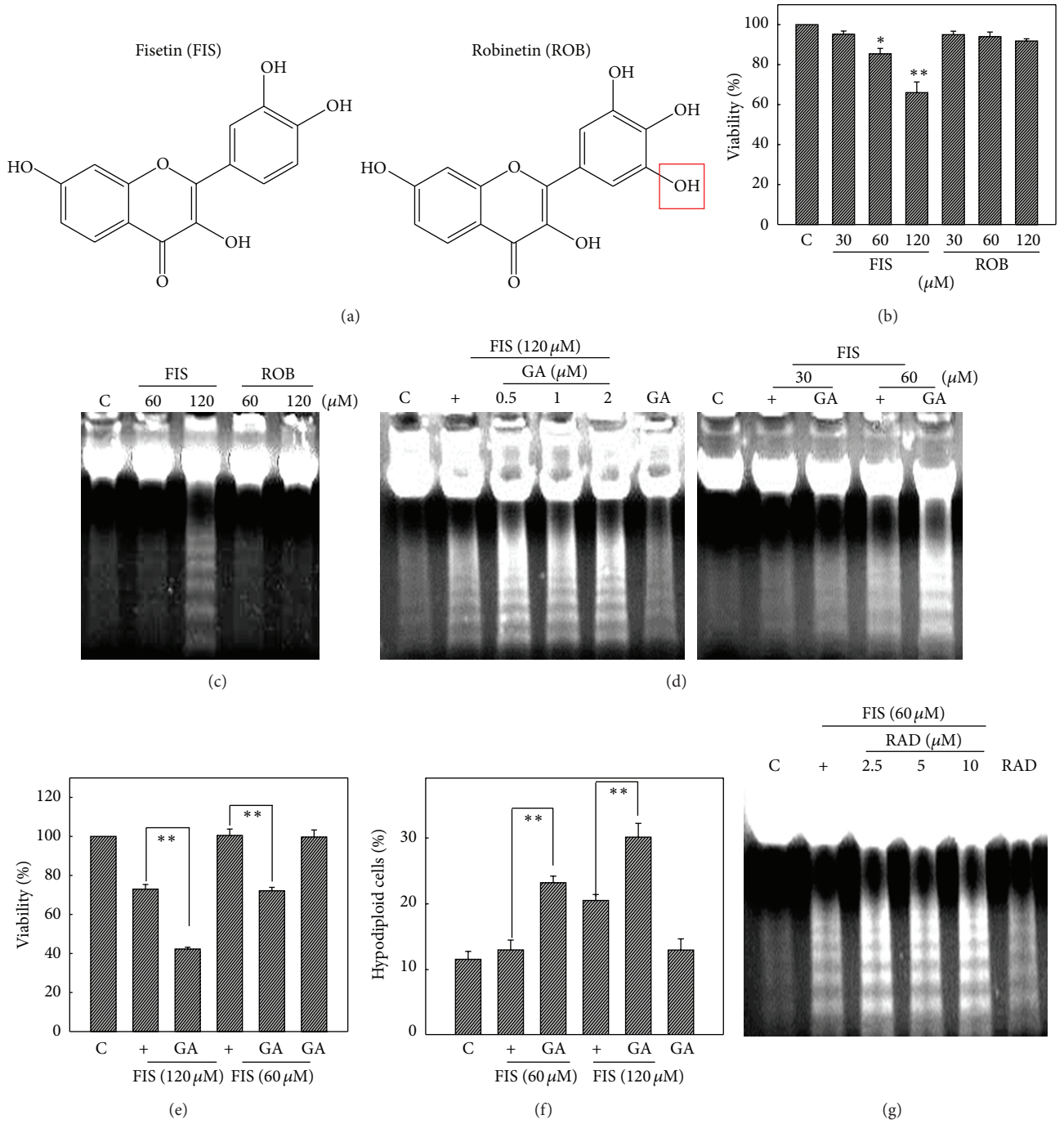

Figure 1: Effects of geldanamycin (GA) and radicicol (RAD) on the viability of COLO205 colorectal carcinoma cells under fisetin (FIS) stimulation. (a) The chemical structures of FIS and the structurally related robinetin (ROB) are depicted. (b) FIS reduction of cell viability of COLO205 cells. COLO205 cells were treated with the indicated concentrations (30, 60, and 120 μM) of FIS for 24 h, and cell viability was examined by an MTT assay. (c) DNA ladders induced by FIS or ROB (60 or 120 μM) in COLO205 cells were detected by agarose electrophoresis. (d) GA enhanced DNA ladder formation in COLO205 cells stimulated by FIS. (Left panel) cells were treated with different concentrations of GA (0.5, 1, and 2 μM) with or without FIS (120 μM) stimulation for 24 h. (Right panel) cells were treated with GA (2 μM) with or without FIS (30 or 60 μM) stimulation for 24 h. The integrity of DNA was analyzed by agarose electrophoresis. (e) GA increased the cytotoxicity of FIS against the viability of COLO205 cells. Cells were treated with GA (2 μM) and FIS (60 or 120 μM) for 24 h, and the viability of cells was examined by an MTT assay. (f) Increases in the percentage of hypodiploid cells by GA in FIS-treated COLO205 cells. As described above, the ratio of hypodiploid cells under different treatments was examined by a flow cytometric analysis using propidium iodide (PI) staining. (g) RAD addition increased the intensity of DNA ladder formation in FIS-treated COLOL205 cells. Cells were treated with different concentrations (2.5, 5, and 10 μM) of RAD with FIS (60 μM) for 24 h, and the integrity of DNA was analyzed. Each data point was calculated from three triplicate groups, and data are displayed as the mean ± S.E. $^{*}P < 0.05$ and $^{**}P < 0.01$ denote a significant difference compared to the control (C) group (Figure 1(b)) or between the indicated groups (Figures 1(e) and 1(f)).

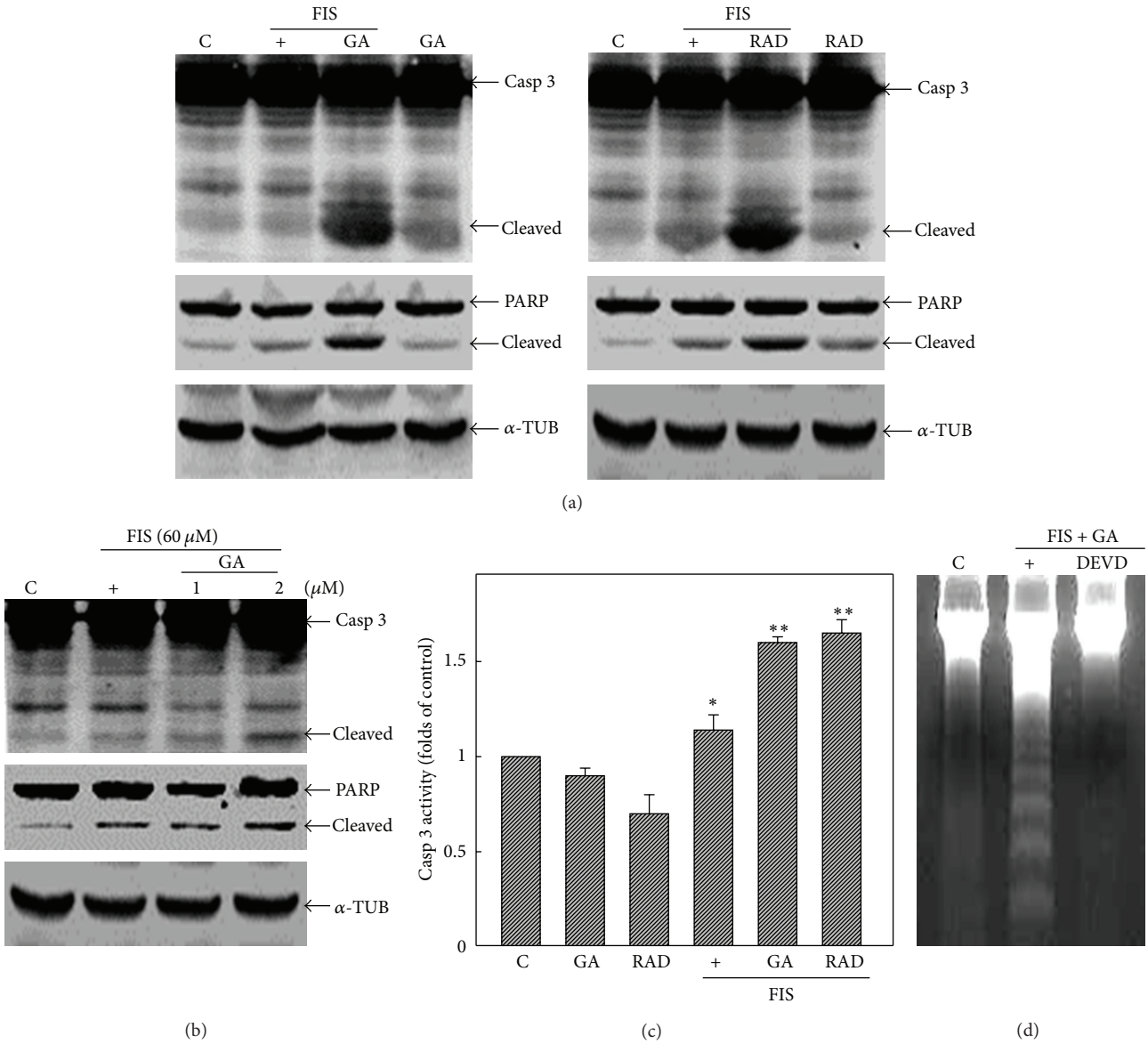

Figure 2: Geldanamycin (GA) and radicicol (RAD) increased caspase-3 and PARP protein cleavage in fisetin- (FIS-) treated COLO205 cells. (a) GA and RAD induced cleavage of caspase-3 and PARP in FIS-treated COLOL205 cells. COLO205 cells were treated with GA (2 μM) or RAD (5 μM) with or without FIS (60 μM) for 24 h, and the expressions of caspase-3, PARP, and α-tubulin protein were detected by western blotting using specific antibodies. (b) Concentration-dependent investigation of GA on caspase-3 and PARP protein cleavage in FIS-treated COLO205 cells. (c) Effects of GA (2 μM), RAD (5 μM), FIS (60 μM), FIS (60 μM)+GA (2 μM), and FIS (60 μM)+RAD (5 μM) on caspase-3 enzyme activity were examined using Ac-DEVD-pNA as a caspase-3-specific peptidyl substrate. Cells were treated with the indicated components for 24 h, and caspase-3 activity was measured using Ac-DEVD-pNA as a substrate. (d) The addition of the caspase-3 peptidyl inhibitor, Ac-DEVD-FMK, inhibited FIS+GA-induced DNA ladder formation in COLO205 cells. Cells were incubated with the caspase-3 inhibitor, Ac-DEVD-FMK, for 2 h, followed by FIS+GA stimulation for additional 24 h, and DNA integrity was analyzed. Each data point was calculated from three triplicate groups, and data are displayed as the mean ± S.E. $^{*}P < 0.05$ and $^{**}P < 0.01$ denote a significant difference from the control (C) group.

decreased to 84% of the initial amount after 8 h of CHX treatment. When COLO205 cells were treated with FIS/CHX, GA/CHX, or RAD/CHX, the stability of the p53 protein did not change, compared to that in CHX-treated COLO205 cells. However, following cotreatment of FIS+GA or FIS+RAD with CHX, the preexisting p53 protein markedly decreased, indicating a protein half-life of around 2 h. This finding indicates that GA decreases p53 protein in FIS-treated COLO205 cells by inducing its degradation.

4. Discussion

According to a systematic review of cohort studies and randomized controlled trials (RCTs), there is insufficient and conflicting evidence regarding the prevention of colorectal neoplasms by flavonoids [15, 16]. The most acceptable reason is that intake of flavonoids at an undetermined dose provides convincing clinical evidence of an anticancer potential [17–19]. However, our investigations disclosed a molecular basis

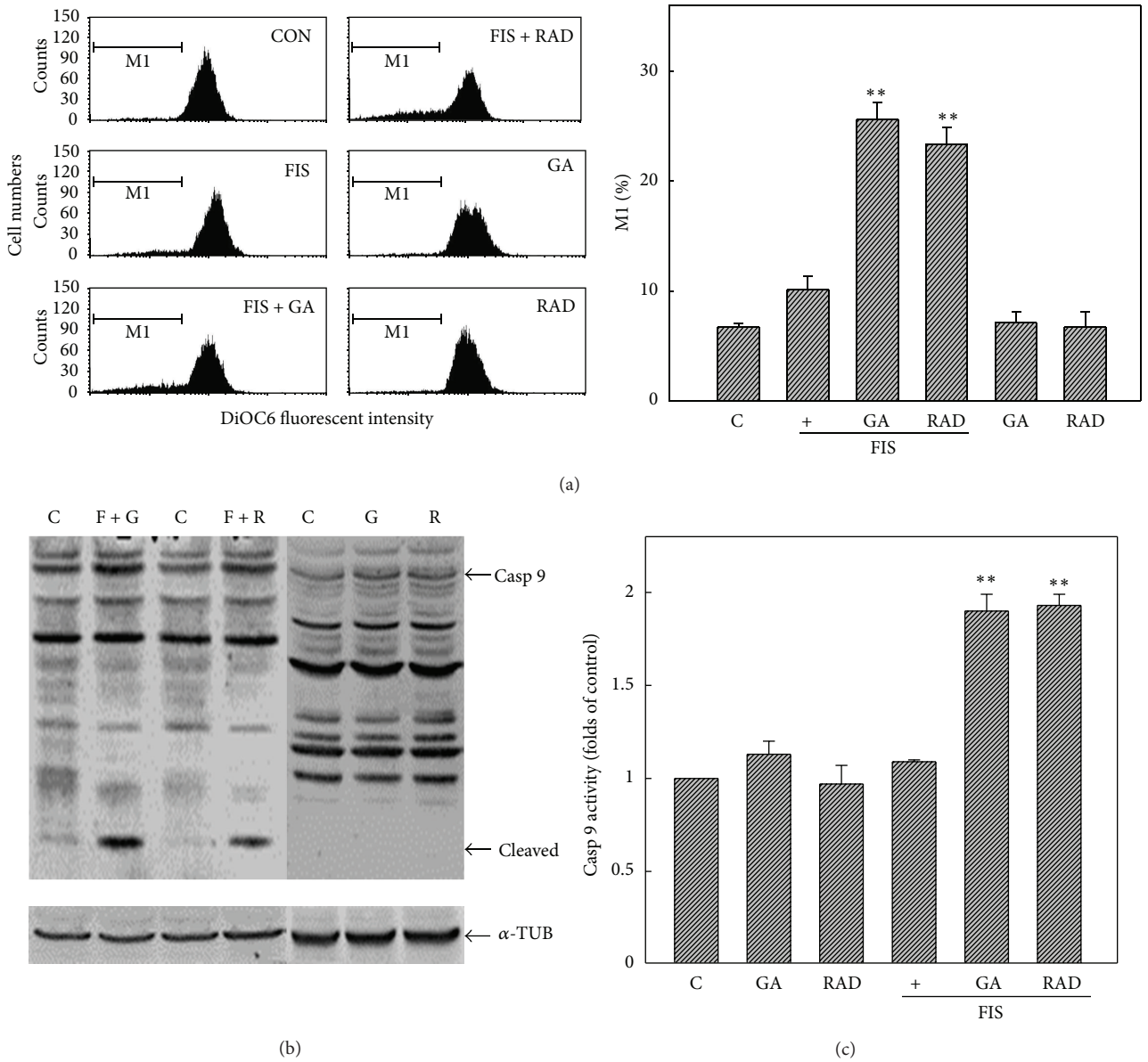

Figure 3: Disruption of the mitochondrial membrane potential (MMP) by fisetin (FIS)+geldanamycin (GA) or FIS+radicicol (RAD) in COLO205 cells. (a) GA or RAD increased loss of the MMP by FIS in COLO205 cells. Cells were treated with GA (G; 2 μM) or RAD (R; 5 μM) with or without FIS (F; 60 μM) for 6 h, and the MMP was detected by a flow cytometric analysis using DiOC6 as a fluorescent dye. (Left panel) a representative of flow cytometric analysis. (Right panel) percentage of M1 was measured and expressed as the mean ± S.E. from three independent experiments. (b) GA or RAD induced cleavage of the caspase-9 protein in FIS-treated COLO205 cells. Cells were treated with GA, RAD, FIS+GA, or FIS+RAD for 24 h, and expression of caspase-9 (Casp 9) protein was detected by western blotting. (c) Increased caspase-9 activity by FIS+GA or FIS+RAD in COLO205 cells. The peptidyl caspase 9 substrate, Ac-YVAD-pNA, was used to detect caspase-9 activity in COLO205 cells under different treatments. Each data point was calculated from three triplicate groups, and data are displayed as the mean ± S.E. $^{**}P < 0.01$ denotes a significant difference from the control (C) group.

for the complementary treatment of human colon cancer with a combination of flavonoids and targeted therapy. The main finding of the present study was that HSP90 inhibitors increased the cytotoxic effect of FIS in colonic carcinoma cells through inducing apoptosis. Induction of caspase-3 activation and reduction of the Bcl-2 protein in accordance with a decreased MMP and decreased p53 protein were detected in COLO205 cells treated with the combination of HSP90 inhibitors and FIS. A strategy to treat colon carcinoma with a combination of FIS and HSP90 inhibitors is suggested.

Caspases are a family of proteases that are the principal executioners of apoptosis, and their cleavage and subsequent activation are considered primary hallmarks of apoptosis. Caspase-3 is a critical executioner of apoptosis by cleavage of several essential cellular proteins such as PARP and D4-GDI. Apoptosis induction through activation of caspase activity by FIS was reported in several previous studies. Lim et al. [7] and Yu et al. [20] reported that FIS induced apoptosis and cleavage of caspases in HCT-116 colorectal carcinoma cells. Ying et al. [21] reported that FIS induced apoptosis in HeLa

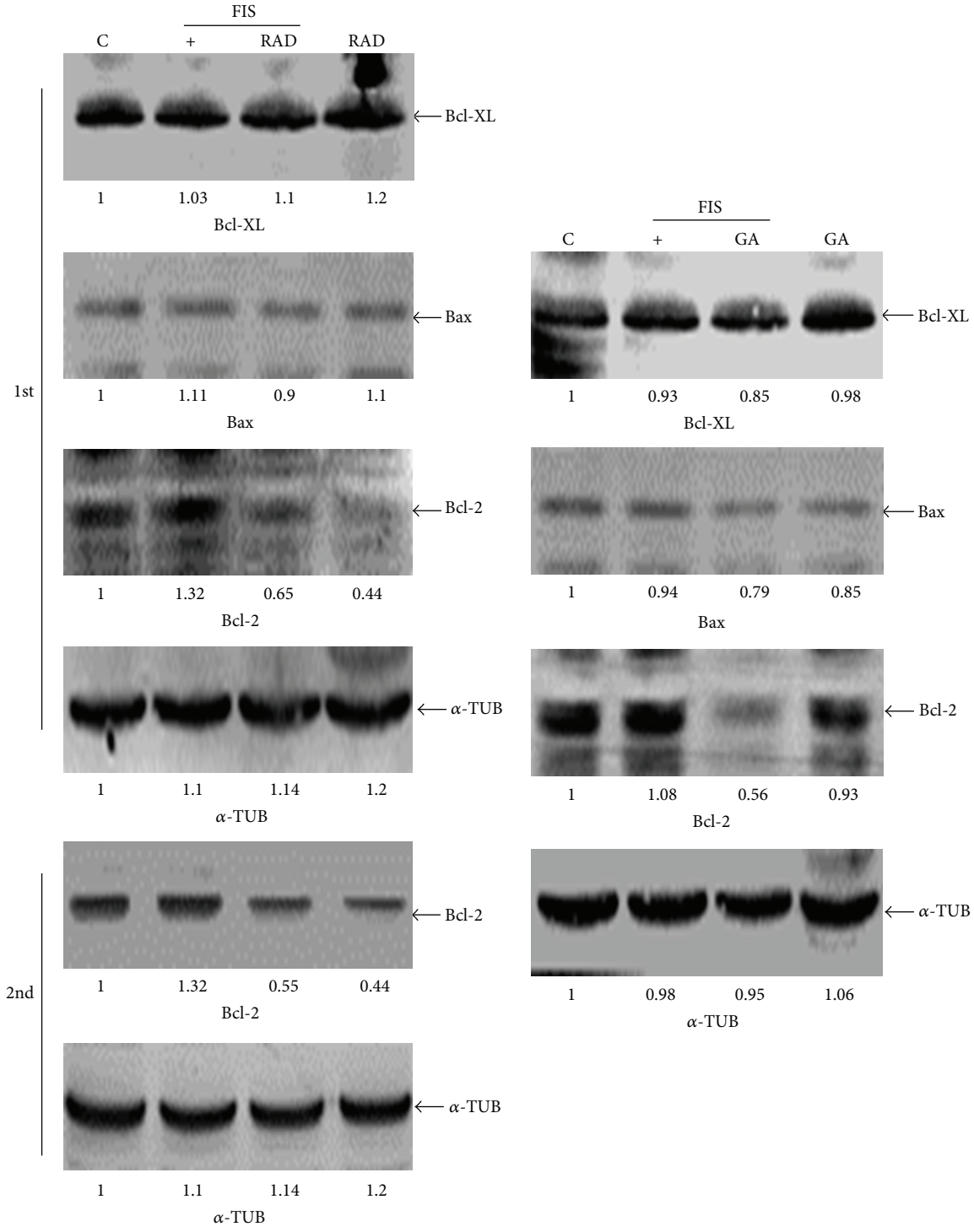

Figure 4: Alternative expression of Bcl-2 family proteins including Bcl-XL, Bcl-2, and Bax in fisetin- (FIS-), geldanamycin- (GA-), and radicicol- (RAD-) treated COLO205 cells. COLO205 cells were treated with FIS (60 μM), GA (2 μM), RAD (5 μM), or their combinations for 24 h, and expressions of the indicated proteins were detected by Western blotting using specific antibodies. Data were repeated at least three times, and similar results were obtained. Data related to RAD-inhibited Bcl-2 protein expression from two independent experiments as labeled in 1st and 2nd were involved. The intensity of each band was examined by a densitometric analysis (ImageJ) and expressed as multiples of the control.

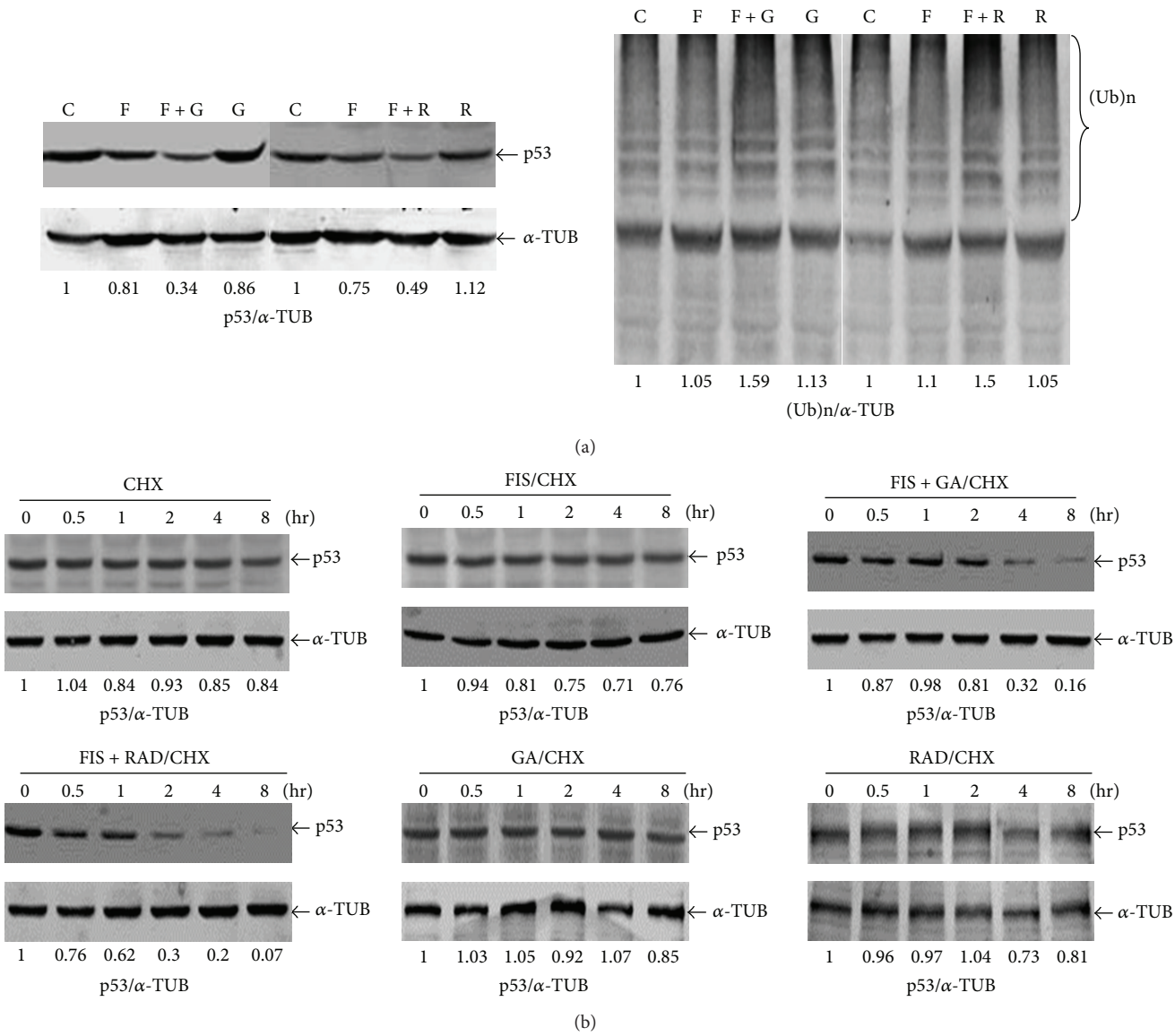

Figure 5: Decreased p53 and its stability, and increased ubiquitination in fisetin (FIS)+geldanamycin (GA)- or FIS+radicicol (RAD)-treated COLO205 cells. (a) Reduction of the p53 protein level and induction of ubiquitin-tagged proteins (Ub) in FIS+GA- or FIS+RAD-treated COLO205 cells. Cells were treated with FIS (60 μM), GA (2 μM), RAD (5 μM), or their combinations for 4 h, and expressions of p53 and Ub-tagged proteins were detected by Western blotting. Intensities of (Ub)n and the p53 protein were quantified, and the ratio of p53/α-TUB and (Ub)n/α-TUB in the respective control group was described as 1. (b) GA and RAD decreased p53 protein stability in FIS-treated COLO205 cells. Cells were treated with cycloheximide (CHX; 1 μg/mL) for 1 h, followed by the addition of FIS (FIS/CHX), FIS+GA (FIS+GA/CHX), FIS+RAD (FIS+RAD/CHX), GA (GA/CHX), or RAD (RAD/CHX) for different times (0, 0.5, 1, 2, 4, and 8 h). At the various time points, expressions of p53 and the α-tubulin protein were detected by western blotting using specific antibodies. Intensities of (Ub)n and the p53 protein were quantified, and the ratio of p53/α-TUB in the respective control group was described as 1. Data were repeated at least three times, and similar results were obtained.

human cervical cancer cells through activation of a caspase-3- and -8-dependent pathway. FIS causes apoptosis in human prostate cancer LNCaP cells through activation of caspases-3, -8, and -9. Among HSP90 inhibitors, GA and RAD are prototypes of 17-N-allylamino-17-demethoxygeldanamycin (17-AAG). Concomitant use of such agents with chemotherapy was suggested to be a potential treatment for cancer [22, 23]. For example, 17-AAG abolished Akt activation and potentiated the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, in breast cancer cells [24]. A combination of 17-AAG and carboplatin remarkably inhibited the growth of human ovarian cancer [25]. In our study, HSP90 inhibitors showed enhancement of the expression of apoptotic proteins in COLO205 cells under FIS stimulation. Caspase-3 activity significantly increased after concurrent treatment with HSP90 inhibitors and FIS, and the effect was diminished by adding a caspase-3 peptidyl inhibitor (Ac-DEVD-FMK). These findings show that activation of caspase

cascades contributes to HSP90- and FIS-induced apoptosis in colon carcinoma cells.

The p53 protein regulates several important cellular functions, including apoptosis, cell-cycle progression, and DNA repair. p53 levels are mainly regulated at the posttranslational level by the ubiquitin-proteasome pathway, and wild-type (WT) p53 was shown to interact with HSP90 [26, 27]. The extended interaction of WT p53 with HSP90 appears to protect the p53 protein from proteolytic degradation, leading to a prolonged half-life. However, the more stable associations of p53 mutants with HSP90 leading to their misfolded conformations were reported. In the present study, the HSP90 inhibitors, GA and RAD, reduced the expression of p53 with an increase in ubiquitination activity in FIS-treated COLO205 cells. Analysis of the p53 half-life showed that the stability of the p53 protein decreased after adding FIS+GA or FIS+RAD. This indicates that enhancement of FIS-induced apoptosis by GA and RAD is mediated by disruption of p53 stability and a reduction in its half-life. Several studies indicated that COLO205 cells express WT p53 [28, 29]. These data suggest that the HSP90 inhibitors, GA and RAD, might enhance the degradation of the WT p53 protein via stimulation of protein ubiquitination to promote apoptosis in COLO205 cells.

HSP90 is involved in the establishment of cancer, and it is overexpressed in various tumors including colon, ovarian, endometrial, gastric, and pancreatic carcinomas [30, 31]. Recent data showed that HSP90 plays an essential role in facilitating the malignant transformation of tumors, is closely related to the increased proliferative potential of cancer cells, and permits tumor cells to escape apoptosis. The oncogenic effects of HSP90 provide an attractive target for treating cancer, and several HSP90 inhibitors, such as GA and RAD, were developed for in vitro and in vivo studies. Our previous study indicated that GA and RAD potentiate apoptosis in amyloid β-treated cerebral blood cells [32]. Misso et al. reported that the combination of tipifarnib with GA induced apoptosis in advanced head and neck squamous cell cancer [33]. Restall and Lorimer showed that GA and RAD can induce premature senescence via apoptosis in small-cell lung cancer cells [34]. Consistent with these data, GA and RAD produced enhanced FIS-induced cell death via apoptosis in COLO205 colon carcinoma cells. This suggests that a combination of HSP90 inhibitors with FIS could be a suitable therapeutic strategy for treating colon carcinoma. In intrinsic apoptotic pathway via disrupting mitochondrial functions, activation of caspase-9 is regulated by members of Bcl-2 family. It is known that decreased antiapoptotic Bcl-2-family proteins such as Bcl-2 and increased proapoptotic Bcl-2-family proteins such as Bax lead to apoptosis. Our results showed that FIS+GA and FIS+RAD can cause a decrease in Bcl-2 protein with an increase in the cleavage of caspase-9 protein, indicating that FIS+GA and FIS+RAD can activate intrinsic mitochondrial apoptotic pathway. An interesting observation is that RAD showed inhibitory effect on Bcl-2 protein expression without affecting caspase-9 activity and mitochondrial membrane potential in COLO205 cells. Yang et al. reported that RAD and GA reduced viability of ovarian carcinoma cells with a decrease in Bcl-2 protein level [35]. Further studies are needed to elucidate the mechanism of RAD-inhibited Bcl-2 protein level in cancer cells.

Taken together, our data reveal that the HSP90 inhibitors, GA and RAD, enhanced the cytotoxicity of FIS via activation of a mitochondria-dependent caspase-3 cascade and accelerated the degradation of the p53 protein. The mechanism of GA and RAD regulated p53-chaperon interactions, and the influence of GA and RAD on HSP90's chaperon function requires further investigation. Our findings highlight the potential of GA and RAD in combination with anticancer drugs, such as FIS, for further in vivo treatment of colon carcinoma.

Abbreviations

FIS:	Fisetin
HSP90:	Heat shock protein 90
GA:	Geldanamycin
RAD:	Radicicol
BCIP:	5-Bromo-4-chloro-3-indolyl phosphate
Bcl-2:	B-cell lymphoma 2
Ub:	Ubiquitin
DCFH-DA:	2′,7′-Dichlorofluorescein diacetate
MMP:	Mitochondrial membrane potential
MTT:	3-(4,5-Dimethylthiazol)-2-yl-2,5-diphenyltetrazolium bromide
NBT:	Nitroblue tetrazolium
PARP:	Poly(ADP-ribose)polymerase
Casp 3:	Caspase-3
Casp 9:	Caspase-9.

Conflict of Interests

None of authors has any conflict of interests related to the study.

Acknowledgments

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