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ORIGINAL RESEARCH

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Comparative evaluation of anti-inflammatory activity of *Manahshila* (Realgar)

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ABSTRACT

Background: Realgar popularly known as *Manahshila* (in Ayurveda) is an arsenic and sulphur containing mineral drug. The *Manahshila* is used after *Shodhana* (purification) for the treatment of various diseases in traditional Indian systems of medicine which also includes pain-related disorders.

Aim: This study is aimed to evaluate the comparative anti-inflammatory efficacy of *Manahshila* using *in vivo* models of inflammation in rats.

Materials and Methods: In the present study, *Shodhana* of *Manahshila* is performed by the *Bhavana* (levigation) where trituration of *Manahshila* is done with ginger (*Zingiber officinale* Rosc.) juice, sesbania leaves (*Sesbania grandiflora* Poir.) juice, lemon (*Citrus medica* Linn.) juice, and lime water. The anti-inflammatory activity of the *Manahshila* at 0.35, 0.7, and 1.4 mg/kg, p.o. was evaluated by egg albumin-induced hind paw edema in rats.

Results: The *Ashodhita Manahshila* (crude realgar) showed mortality, while *Shodhita Manahshila* (purified with all four media) did not showed mortality in the treated rats up to a dose of 2,000 mg/kg. *Ashodhita* and *Shodhita Manahshila* revealed that at the dose level of 1.4 mg/kg it showed inhibition ($p < 0.05$) of egg albumin-induced hind paw edema. But, *Shodhita Manahshila* showed anti-inflammatory activity at all three dose levels in a dose-dependent manner. The ginger juice treated *Manahshila* (56.60%) was found best alleviates rat's response to egg albumin-induced inflammation among all the group.

Conclusion: The results validate its usage in the healing of inflammation and also give support to its perspective as the source of novel pain relief medicine prototype.

ARTICLE HISTORY

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KEYWORDS

Inflammation; *Manahshila*; organomineral; *Shodhana*

Introduction

Inflammation is one of the commonest symptoms of all the arthritic conditions [1]. Non-steroidal anti-inflammatory drugs (NSAIDs) are the drug of choice for such types of conditions. NSAIDs typically alleviate inflammation and allied pain by inhibiting cyclooxygenase enzymes (COX) involved in the production of prostaglandins. Compounds that inhibit COX enzymes could, therefore, be considered to be the potential anti-inflammatory drugs. However, many of the commonly used anti-inflammatory agents is evident with serious adverse reactions such as gastric intolerance, bone marrow depression, water and salt retention, resulting from prolonged use [2]. NSAIDs chiefly corticosteroids and anti-histaminics are being used till now but the potential side effect gives a limitation for their use [3]. Presently, it is a growing attention all over for the improvement of new in noxious and effective, less toxic anti-inflammatory medications. Hence, there is a call to examine for more naturally available alternatives for the management of inflammation [4]. Metals and minerals, i.e., arsenic, lead, and mercury which are used in traditional Ayurvedic medicines are raising

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more concern regarding their toxic effect instead of huge therapeutic panorama [5,6]. But, there are several types of research which proves that addition of minerals in the formulation is based on defined therapeutic properties [7]. The arsenic group of minerals (sulfdes and oxides of arsenic) is considered as toxic but the judicial use of these minerals makes them highly effective. *Manahshila* (disulfide of arsenic, As_2S_2) is a mineral drug belonging to the group of *Uparasa Varga* (an Ayurvedic set of mineral drugs). Commonly crude *Manahshila* (realgar) is very much toxic (LD_{50} 3.2 gm/kg) and carcinogenic [8]. Its solubility and bioavailability are found to be very poor. Inspite of all these factual descriptions, *Manahshila* has long been used in more than 100 formulations in Indian system of medicine for the treatment of various diseases such as *Kasa* (bronchitis), *Swasa* (dyspnea), *Hikka* (hiccough), *Chardi* (emesis), *Kustha* (leprosy and other dermatoses), etc. *Manahshila* is a compound of arsenic and sulfur [9]. In the ancient Ayurvedic treatises, it has been mentioned that any toxic drugs may be converted into safe and effective by using definite pharmaceutical process like *Shodhana* (purification), *Marana* (incineration), etc [10]. By adopting unique procedures described in classics of Ayurveda, changes in the physicochemical characteristics takes place as well as the processed metal and minerals also becomes digestible by the tissue and then the potency/therapeutic efficacy improves [11]. However, depending upon the nature of the minerals and their uses on specific disease, the specific process for purification is selected. In classics, different purification processes and media are described for *Manahshila*. So in the present study, ginger (*Zingiber officinale* Rosc.) juice, sesbania (*Sesbania grandiflora* Poir.) juice, lemon (*Citrus medica* Linn.) juice, and lime water (*Churnodaka*), were taken for *Shodhana* of *Manahshila* by following the process of trituration as mentioned in various alchemical treatises [12,13]. Afterward, a comparative study was performed to find out the effects of various above-mentioned *Shodhana* media, i.e., ginger juice, sesbania juice, lemon juice, and lime water, on the anti-inflammatory activity of *Manahshila* in albino rats.

Subjects and Methods

Procurement of raw materials

Manahshila (95% w/v purity) was acquired from Sigma Aldrich Co. (519111-25G; St. Louis, MO). The plant ingredients (*Z. officinale*, *C. medica*, and *S. grandiflora*) and lime powder were obtained from the herbal market at Gola Deenanath, Varanasi. The voucher specimen (APRIL/HERB/15-16/03-05) of medicinal plants has been stored at the Department of Ayurvedic Pharmacy Research Laboratory, RGSC, Barkachha, Mirzapur, UP, India for further reference.

Drugs and chemicals

The indomethacin (standard drug) was obtained from IPCA Pharmaceuticals, Mumbai, India. All the additional drugs and chemicals were of analytical grade and acquired from S.D. fine Chemicals Pvt. Ltd., Mumbai, India.

Shodhana *(Purification) of* Manahshila

Shodhana of *Manahshila* was performed by the process of *Bhavana* (levigations/wet trituration). *Ashodhita Manahshila* (crude realgar) was taken inside a clean mortar-pestle and made them finely powdered. Next, it was triturated with ginger juice, sesbania juice, lemon juice, and lime water individually for seven times each except for lime water (next two times) [12]. All five samples were coded as *Ashodhita Manahshila* (AM), zinger juice treated *Manahshila* (ZM), sesbania juice treated *Manahshila* (SM), lemon juice treated *Manahshila* (LEM), and lime water treated *Manahshila* (LIM) (Fig. 1).

Dose calculation

Human dose of *Manahshila* is being given as 3.9–7.8 mg, which is the superior dose as has been suggested by ancient alchemists [12]. The highest dose has been considered for the experimental purpose. This was changed into animal dose based on Paget and Barne's surface area ratio [14] which works out to be 0.7 mg/kg body weight.

$$\text{i.e., Rat dose/kg body wt.} = 0.018 \times \text{Human dose} \times 5$$
$$= 0.018 \times 7.8\text{mg} \times 5$$
$$= 0.7 \text{ mg/kg body weight.}$$

Therefore, the experiment was conducted by taking the sample of different concentration such as 0.35, 0.7, and 1.4 mg/kg of body weight of rats to study the anti-inflammatory activity of the drug at this concentration.

Preparation of drug

The required dose of *Manahshila* was prepared by triturating the accurately weighed quantity of the *Manahshila* against each subject body weight, with 0.5% solution of *carboxymethyl cellulose* (CMC) for oral route of administration to test group. Indomethacin was dissolved in CMC solution for oral route administration.

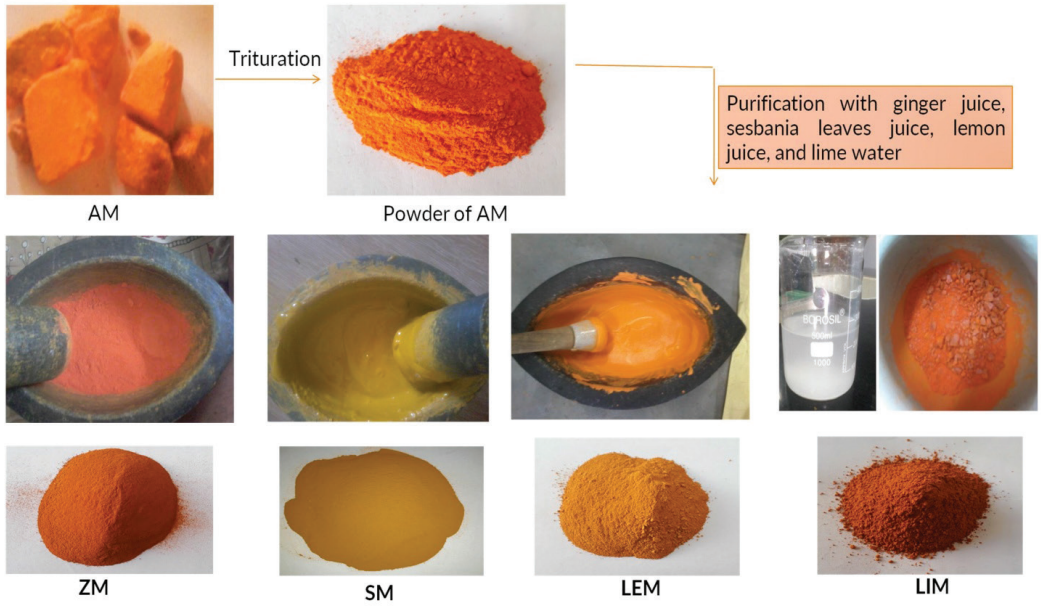

Figure 1. *Shodhana* of *Manahshila* with different media.

Pharmacological evaluation

Animals

Healthy Charles Foster albino rats (160–200 g) of either sex were used for this study. Animals were obtained from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University, Varanasi. The animals were housed in big polypropylene cages at a controlled room temperature (22°C ± 2°C). Humidity (55% ± 10%) was sustained properly and 12 hours light and 12-hour dark cycle were also followed. The animals were provided with standardized pellet feed (Amrut Pvt. Ltd) and fresh drinking water. Rats were acclimatized to the standard laboratory condition for at least 1 week before starting the experiment. Body weight of rats was measured regularly. The principle of laboratory animal care guidelines (NIH publication number 85-23, revised 1985) was followed. Before conduction of the study, approval from the Institutional Animal Ethical Committee (IAEC) for the reason of control and supervision of experiments on animals (Dean/2015/CAEC/1430) had already been taken.

Route of administration

All the drugs were prepared in CMC, whereas the control group received CMC. The drug was administered by oral route daily using a syringe with a curved feeding needle since it is easiest and less traumatic for passage down the esophagus. No food or water was given up to 4 hours after drug treatment.

Organoleptic characterization

Organoleptic characteristics lead to the evaluation of drug by different parameters like color, taste, texture, odor, etc. The organoleptic characteristics of the AM, ZM, SM, LEM, and LIM were carried out based on the process specified in Ayurvedic Pharmacopoeia of India (API) [15].

Acute toxicity study of *Ashodhita* and *Shodhita Manahshila*

The acute toxicity study was conducted out using the Organization for Economic Co-operation and Development Guidelines-425. Totally, juvenile, vigorous, and non-pregnant, Wistar albino female rats, weighing 160–200 g were chosen and acclimatized for 7 days before the experiment. The entire five-test drug along with adjuvant was given to overnight starved rats at graded doses by serving Up and Down method with 2,000 mg/kg. The rats were examined strictly for behavioral changes, signs of toxicity, and fatality if any, continuously for the initial 6 hours, and thereafter periodically up to 14 days [16].

Pharmacological activities

Egg albumin-induced hind paw edema

The anti-inflammatory study was carried out using the method with slight modification in rats [17]. For this study, rats (n = 6) were distributed into 17 groups, where group 1 was served as vehicle control and was administered 0.5% CMC. Group 2 was treated with

standard indomethacin at 10 mg/kg, p.o. while group 3–5, 6–8, 9–11, 12–14, and 15–17 were treated with AM, ZM, SM, LEM, and LIM at 0.35, 0.7, and 1.4 mg/kg p.o. body weight, respectively (Table 1). After 30 minutes of drug administration, rats were injected with 0.1 ml egg albumin into the plantar tissue of the right hind paw. The contra-lateral hind paws of rats were injected with 0.5% CMC as a control. Paw volume was measured plethysmographically at 0, 30th, 60th, 90th, 120th, 150th, 180th, 210th, and 240th minute after egg albumin injection. The level of inhibition of edema was calculated for *Ashodhita* and *Shodhita* drug using the relation.

$$\% \text{ Inhibition} = \frac{\text{increase in paw edema (control)} - \text{increase in paw edema (test)}}{\text{Increase in paw edema (control)}} \times 100$$

Statistical analysis

To examine the significant effect of drugs and doses on the rats, we analyzed the data that were expressed as mean ± SEM (n = 6 animals in each one group) by two-way analysis of variance (ANOVA) followed by Tukey test performed at 95% level of significance using GraphPad InStat version 3.00, GraphPad Software, San Diego, California, www.graphpad.com and for the computations we used the software R (www.r-project.org). p value was considered as significant when p <0.05 at 95% confidence level.

Results

Organoleptic characterization

The color of AM, ZM, SM, LEM, and LIM have been examined and it has showed orange, reddish yellow, greenish yellow, sunflower, and sienna, respectively. The texture of all the five powdered samples was found to be smooth and fines while the odor of AM and LIM found odorless. The characteristic (aromatic, pungent) odor presented in the ZM, SM, and LEM (Table 2).

Acute oral toxicity study

The acute oral toxicity study showed that ZM, SM, LEM, and LIM up to 2,000 mg/kg body weight did not show any sign and symptoms of toxicity and mortality. While AM showed sign and symptoms of toxicity and mortality like trembling, piloerection, fore-limbs, body extended, breathing complexity, etc.

Effects of AM, ZM, SM, LEM, and LIM on egg albumin test

The results of egg albumin test have been summarized in Table 3–7. At the dose level of 1.4, 0.7 mg/kg, and 0.35 p.o. the AM, ZM, SM, LEM, and LIM showed more significant suppression of egg white-induced edema compared with control group ($p < 0.05$, $p < 0.01$, or $p < 0.001$) at 30th, 60th, 90th, 120th, 150th, 180th, 210th, and 240th minute of treatment. While maximum activity was found in ZM and SM at 240 minutes. While the effect of LEM and LIM exhibited a significant suppression on paw edema in rats than that of AM. The standard drug indomethacin (10 mg/kg, p.o.) showed significant reduction in hind paw volume after 180th, 210th, and 240th minute ($p < 0.05$).

From Tables 8 and 9, at 5% level of significance, we conclude that the effect of doses and drugs are independent and there is significant effect of doses and drugs on time of observations (Fig. 2-4). As at

Table 1. Egg albumin-induced hind paw edema

NC(Egg Albumin) (mg/kg, i.p.)	IND (mg/kg, p.o.)	AMtreated (mg/kg, p.o.)	ZMtreated (mg/kg, p.o.)	SM treated (mg/ kg, p.o.)	LEMtreated (mg/ kg, p.o.)	LIMtreated (mg/ kg, p.o.)
0.1	10	0.35	0.35	0.35	0.35	0.35
-	-	0.7	0.7	0.7	0.7	0.7
-	-	1.4	1.4	1.4	1.4	1.4

NC = Negative control, IND = Indomethacin.

Table 2. Organoleptic characteristics of *Ashodhita* and *Shodhita Manahshila.*

Drug	Color	Texture	Odor
AM	Orange	Smooth, fine	Odorless
ZM	Reddish orange	Smooth, fine	Characteristic
SM	Greenish yellow	Smooth, fine	Characteristic
LEM	Sunflower	Smooth, fine	Characteristic
LIM	Sienna	Smooth, fine	Odorless

Table 3. Anti-inflammatory activity of the *Ashodhita Manahshila* of egg albumin-induced paw edema in albino rats.

	30 minutes	60 minutes	90 minutes	120 minutes	150 minutes	180 minutes	210 minutes	240 minutes
NC	0.399 ± 0.005	0.628 ± 0.010	0.798 ± 0.012	0.853 ± 0.010	1.054 ± 0.013	1.091 ± 0.010	1.05 ± 0.010	0.924 ± 0.012
IND (10mg/kg)	0.212 ± 0.004[a]	0.297 ± 0.008[a]	0.365 ± 0.005[a]	0.390 ± 0.012[a]	0.412 ± 0.008[a]	0.390 ± 0.012[a]	0.342 ± 0.008[a]	0.282 ± 0.008[a]
AM (1.4 mg/kg)	0.371 ± 0.012[ab]	0.512 ± 0.014[ab]	0.536 ± 0.015[ab]	0.608 ± 0.010[ab]	0.624 ± 0.012[ab]	0.615 ± 0.011[ab]	0.667 ± 0.014[ab]	0.635 ± 0.012[ab]
AM (0.7 mg/kg)	0.387 ± 0.011[ac]	0.556 ± 0.008[ac]	0.571 ± 0.011[ac]	0.657 ± 0.014[ac]	0.709 ± 0.014[ac]	0.721 ± 0.013[ac]	0.708 ± 0.012[ac]	0.668 ± 0.013[ac]
AM (0.35 mg/kg)	0.392 ± 0.015[ac]	0.578 ± 0.011[ac]	0.595 ± 0.010[ac]	0.782 ± 0.014[ac]	0.784 ± 0.013[ac]	0.755 ± 0.011[ac]	0.720 ± 0.014[ac]	0.715 ± 0.014[ac]

Table 4. Anti-inflammatory activity of the ginger juice treated *Manahshila* of egg albumin-induced paw edema in albino rats.

	30 minutes	60 minutes	90 minutes	120 minutes	150 minutes	180 minutes	210 minutes	240 minutes
NC	0.399 ± 0.005	0.628 ± 0.010	0.798 ± 0.012	0.853 ± 0.010	1.054 ± 0.013	1.091 ± 0.010	1.05 ± 0.010	0.924 ± 0.012
IND (10 mg/kg)	0.212 ± 0.004[a]	0.297 ± 0.008[a]	0.365 ± 0.005[a]	0.390 ± 0.012[a]	0.412 ± 0.008[a]	0.390 ± 0.012[a]	0.342 ± 0.008[a]	0.282 ± 0.008[a]
ZM (1.4 mg/kg)	0.258 ± 0.007[ab]	0.355 ± 0.010[ab]	0.417 ± 0.009[ab]	0.447 ± 0.012[ab]	0.472 ± 0.006[ab]	0.442 ± 0.015[ab]	0.425 ± 0.01[2ab]	0.401 ± 0.012[ab]
ZM (0.7 mg/kg)	0.297 ± 0.004[ab]	0.410 ± 0.008[ab]	0.452 ± 0.010[ab]	0.498 ± 0.010[ab]	0.541 ± 0.014[ab]	0.588 ± 0.005[ab]	0.501 ± 0.007[ab]	0.465 ± 0.015[ab]
ZM (0.35 mg/kg)	0.334 ± 0.008[ac]	0.431 ± 0.009[ac]	0.476 ± 0.009[ac]	0.522 ± 0.007[ac]	0.553 ± 0.008[ac]	0.564 ± 0.007[ac]	0.542 ± 0.011[ac]	0.520 ± 0.008[ac]

Table 5. Anti-inflammatory activity of the sesbania leaves juice treated *Manahshila* of egg albumin-induced paw edema in albino rats.

	30 minutes	60 minutes	90 minutes	120 minutes	150 minutes	180 minutes	210 minutes	240 minutes
NC	0.399 ± 0.005	0.628 ± 0.010	0.798 ± 0.012	0.853 ± 0.010	1.054 ± 0.013	1.091 ± 0.010	1.05 ± 0.010	0.924 ± 0.012
IND (10 mg/kg)	0.212 ± 0.004[a]	0.297 ± 0.008[a]	0.365 ± 0.005[a]	0.390 ± 0.012[a]	0.412 ± 0.008[a]	0.390 ± 0.012[a]	0.342 ± 0.008[a]	0.282 ± 0.008[a]
SM (1.4 mg/kg)	0.298 ± 0.005[ab]	0.392 ± 0.014[ab]	0.443 ± 0.005[ab]	0.482 ± 0.009[ab]	0.494 ± 0.011[ab]	0.501 ± 0.010[ab]	0.481 ± 0.005[ab]	0.442 ± 0.014[ab]
SM (0.7 mg/kg)	0.320 ± 0.004[ab]	0.441 ± 0.009[ab]	0.467 ± 0.016[ab]	0.527 ± 0.012[ab]	0.562 ± 0.006[ab]	0.547 ± 0.013[ab]	0.523 ± 0.010[ab]	0.501 ± 0.010[ab]
SM (0.35 mg/kg)	0.351 ± 0.010[ac]	0.465 ± 0.013[ac]	0.518 ± 0.010[ac]	0.568 ± 0.010[ac]	0.581 ± 0.011[ac]	0.594 ± 0.011[ac]	0.566 ± 0.015[ac]	0.542 ± 0.013[ac]

Table 6. Anti-inflammatory activity of the lemon juice treated *Manahshila* of egg albumin-induced paw edema in albino rats.

	30 minutes	60 minutes	90 minutes	120 minutes	150 minutes	180 minutes	210 minutes	240 minutes
NC	0.399 ± 0.005	0.628 ± 0.010	0.798 ± 0.012	0.853 ± 0.010	1.054 ± 0.013	1.091 ± 0.010	1.05 ± 0.010	0.924 ± 0.012
IND (10mg/kg)	0.212 ± 0.004[a]	0.297 ± 0.008[a]	0.365 ± 0.005[a]	0.390 ± 0.012[a]	0.412 ± 0.008[a]	0.390 ± 0.012[a]	0.342 ± 0.008[a]	0.282 ± 0.008[a]
Lemon (1.4 mg/kg)	0.317 ± 0.010[ab]	0.417 ± 0.004[ab]	0.452 ± 0.008[ab]	0.510 ± 0.010[ab]	0.531 ± 0.006[ab]	0.511 ± 0.014[ab]	0.517 ± 0.013[ab]	0.491 ± 0.009[ab]
Lemon (0.7 mg/kg)	0.345 ± 0.008[ab]	0.450 ± 0.009[ab]	0.497 ± 0.015[ab]	0.547 ± 0.015[ab]	0.597 ± 0.015[ab]	0.582 ± 0.006[ab]	0.569 ± 0.010[ab]	0.542 ± 0.016[ab]
Lemon (0.35 mg/kg)	0.368 ± 0.007[ac]	0.478 ± 0.011[ac]	0.536 ± 0.010[ac]	0.593 ± 0.014[ac]	0.611 ± 0.012[ac]	0.634 ± 0.010[ac]	0.591 ± 0.015[ac]	0.578 ± 0.013[ac]

Table 7. Anti-inflammatory activity of the lime-treated *Manahshila* of egg albumin-induced paw edema in albino rats.

	30 minutes	60 minutes	90 minutes	120 minutes	150 minutes	180 minutes	210 minutes	240 minutes
NC	0.399 ± 0.005	0.628 ± 0.010	0.798 ± 0.012	0.853 ± 0.010	1.054 ± 0.013	1.091 ± 0.010	1.05 ± 0.010	0.924 ± 0.012
IND (10 mg/g)	0.212 ± 0.004[a]	0.297 ± 0.008[a]	0.365 ± 0.005[a]	0.390 ± 0.012[a]	0.412 ± 0.008[a]	0.390 ± 0.012[a]	0.342 ± 0.008[a]	0.282 ± 0.008[a]
LIM (1.4 mg/kg)	0.352 ± 0.009[ab]	0.458 ± 0.009[ab]	0.498 ± 0.015[ab]	0.574 ± 0.009[ab]	0.589 ± 0.015[ab]	0.560 ± 0.014[ab]	0.580 ± 0.010[ab]	0.547 ± 0.013[ab]
LIM (0.7 mg/kg)	0.366 ± 0.010[ab]	0.475 ± 0.013[ab]	0.529 ± 0.007[ab]	0.610 ± 0.011[ab]	0.621 ± 0.012[ab]	0.623 ± 0.005[ab]	0.610 ± 0.012[ab]	0.582 ±0 .015[ab]
LIM (0.35 mg/kg)	0.388 ± 0.007[ac]	0.493 ± 0.015[ac]	0.578 ± 0.010[ac]	0.635 ± 0.008[ac]	0.683 ± 0.007[ac]	0.687 ± 0.010[ac]	0.665 ± 0.008[ac]	0.635 ± 0.010[ac]

Values are expressed as mean ± SEM (n = 6). Statistical comparison was analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. [a]$p < 0.05$, statistically significant as compared with negative control; [b]$p < 0.05$, statistically significant as compared with AM, ZM, SM, LEM, and LIM (1.4, 0.7, and 0.35 mg/kg, p.o.). [c]$p < 0.05$, statistically significant as compared with Indomethacin (IND 10 mg/kg, p.o.).

5% level of significance, the critical value of the studentized range is 3.8982 and minimum significant difference is 0.0934, so we conclude that there is a significant difference between AM, SM, and ZM (Table 9).

Discussion

Arsenic (As) has been believed to be a poison for years; moreover, it is usually recognized as a greatly effective environmental co-carcinogen toward human malignancies, mainly for lung and skin cancer [18]. But, the arsenical compounds were found less toxic than arsenic because of sulfur (S) is associated into disulfide linkage in several proteins and acts crucial function in arsenic detoxification [19]. Generally, the toxicity of the arsenical compounds like realgar, orpiment, and white arsenic depended on their exposure dose, age, sex, chemical states, the biological classes, along with on genetic, specific susceptivities, and nutritional factors [20]. *Manahshila* is identified as realgar (Arsenic disulphide As_2S_2). It has been mentioned as one of the efficacious remedy for the treatment of *Kasa*, *Swasa*, *Hikka*, *Chardi*, and *Kustha* in ancient treatises [12]. Metals and minerals used in their raw form are known to possess the capability to cause severe ailments in the human body. Thus, various pharmaceutical precesses, i.e., *Shodhana* (purification) *Marana* (incineration), etc. are described by ancient seers to make these metals and minerals suitable for human consumption. *Shodhana* with different media facilitates the size reduction which increases the particle surface area, enhances safety, potency, and also reduced the toxicity [21]. For *Shodhana* (purification) of *Manahshila*, procedure of *Bhavana* (wet trituration) is used usually. *Bhavana* is wet grinding or trituration by the means of specified liquid media for a specific duration of time, which leads to removal of impurities present in mineral (*Manahshila*) and also leads to impregnation of therapeutic principles of liquid media in the *Mardita Dravya* (*Manahshila*). It can also be assumed that the liquid media not only add their therapeutic properties but also chelated the toxic substances present in the drug material [9]. The various mentioned liquid media such as ginger juice, sesbania juice, lemon juice, and lime water were used in this study [13]. *Shodhana* of *Manahshila* by ginger juice, sesbania leaves juice, and lemon juice involves numerous affirmative features. Detoxification of heavy metals by chelation is done by phytochelatins. These phytochelatins are heavy metal-binding peptides. There are four

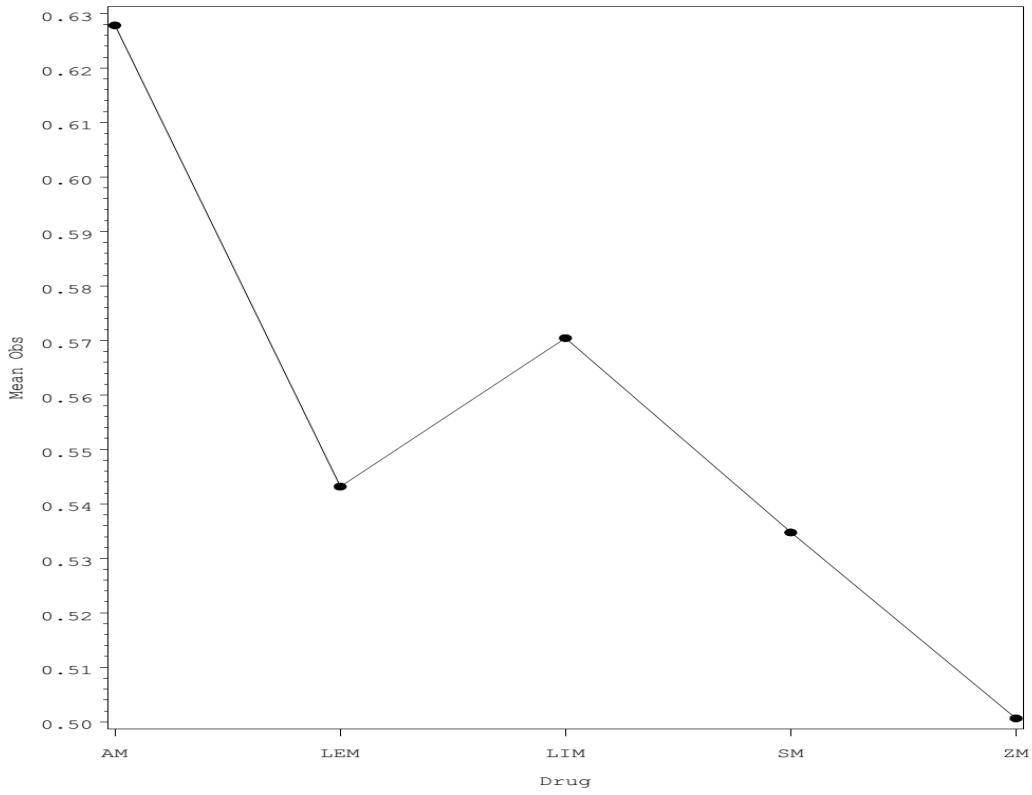

Figure 2. Graph shows the mean effect of the AM, ZM, SM, LEM, and LIM on inflammation.

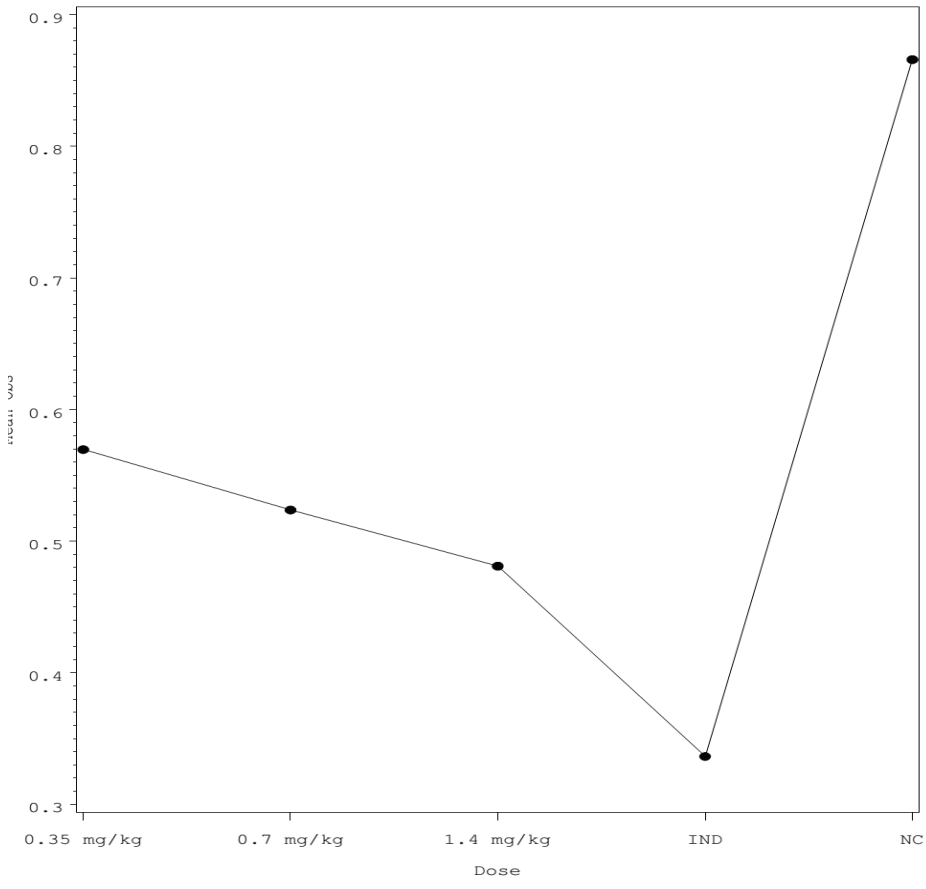

Figure 3. Graph shows the mean effect of different level dose (0.35, 0.7, and 1.4 mg/kg) of drug on inflammation.

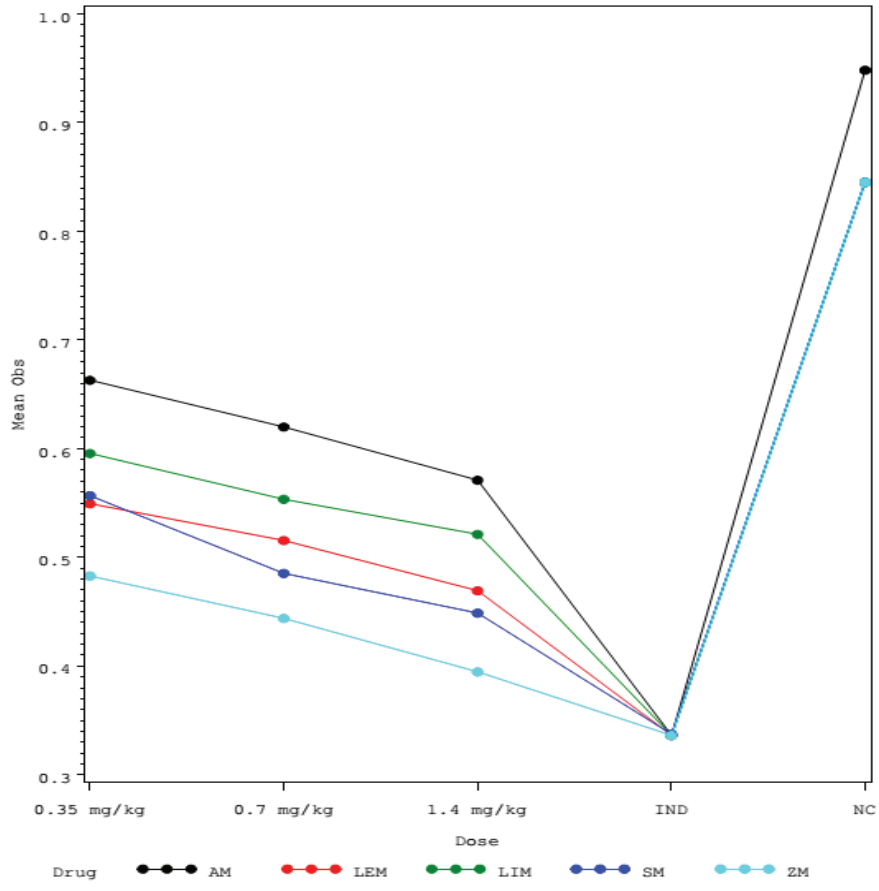

Figure 4. Graph shows the mean effect of the intersection of a different level dose of drug on inflammation.

Table 8. ANOVA table for two-way classified data doses and drug.

Source	DF	SS	Mean Square	*F* Value	Pr > F
Dose	4	6.0407	1.5101	65.74	<0.0001
Drug	4	0.3619	0.0904	3.94	0.0044
Dose*Drug	16	0.1377	0.0086	0.37	0.9867
Residual error	175	4.0199	0.0230		

Table 9. Turkey's studentized range test for drug.

Means with the same letter are not significantly different				
Tukey Grouping		Mean	*N*	Medicine
	A	0.628	40	AM
B	A	0.570	40	LIM
B	A	0.534	40	LEM
B		0.522	40	SM
B		0.500	40	ZM

types of sulfur containing amino acids (taurine, cysteine, homocysteine, and methionine). But, only cysteine and methionine are incorporated into proteins [22]. *Manahshila* may be turned into arsenic nontoxic by these phytochelatins. Methylation is facilitated by methyl donor peptide in ginger known as cysteine. By the methylation process, all the *Shodhana* media (except lime water) detoxifying of arsenic in the body through hastened excretion. In this process, addition of a methyl group to

the arsenic and alters it into a nontoxic form in the liver is taken by this process then excreted out [23]. All the *Shodhana* media (except lime water) act as preservation of glutathione, which is an important detoxifying compound present in the blood. It also acts as natural antioxidant recycling enzyme which unites with arsenic and excretes it through the bile. The level of glutathione is reduced by arsenic poisoning in the blood. The purification media may be acts as a cumulative approach, which increased the amount of glutathione in the blood [24–26]. Natural *Manahshila* occurs mainly in hydrothermal and magmatic ore places. They generally exist in the mixture of arsenic trioxide (As_2O_3) and arsenic sulfide ($As_2 S_2$). Arsenic trioxide is known to be more toxic in nature as compared to arsenic sulphide [27]. Lime water is generally used for *Shodhana* because white arsenic trioxide readily dissolves in a solution of alkali, but arsenic sulfide is insoluble in alkaline solution. Here, lime water acts to eradicate highly toxic arsenic trioxide from *Manahshila* [21]. In this article, emphasis has been given to find out the anti-inflammatory property of *Manahshila* by using four different liquid media and further to evaluate the maximally effective liquid media for the above-mentioned therapeutic effect. The anti-inflammatory activity of *Manahshila* was evaluated before and after the treatment with different *Shodhana* media (juice of ginger rhizome, juice of sesbania leaves, lemon juice, and lime water) in rats at the dose of 1.4, 0.7, and 0.35 mg/kg p.o. in egg albumin-induced inflammatory model (Table 1). In the earlier report, it was found that all the *Shodhana* media was found anti-inflammatory property by inhibiting prostaglandin and leukotriene biosynthesis to the elimination of 5-lipoxygenase or prostaglandin synthetase. The lavish production of pro-inflammatory cytokines (NO, IL-1, TNF-α, and IL-8) were also inhibited by these constituents [28,29]. In another study, it was also showed that the extract of ginger juice decreased the elevated character of NFκB and TNF-α [30–34]. Anti-inflammatory activities of *Ashodhita* and *Shodhita Manahshila* were performed on egg albumin-induced rats hind paw edema. *Ashodhita* and *Shodhita Manahshila* showed significant activity on egg albumin models of inflammation. The *Shodhita Manahshila* (1.4, 0.7, and 0.35 mg/kg, p.o.) reduced inflammation in egg albumin-induced inflammation model in a dose-dependent manner. The hind paw volume in egg albumin-induced edema in rats treated with standard drug, AM (1.4, 0.7, and 0.35 mg/kg, p.o. at 240 minutes) was found to be 0.282 ± 0.008, 0.635 ± 0.012, 0.668 ± 0.013, and 0.715 ± 0.014 ml, respectively. The ZM (1.4, 0.7, and 0.35 mg/kg, p.o. at 240 minutes) was found to be 0.401 ± 0.012, 0.465 ± 0.015, and 0.520 ± 0.008 ml, respectively. The SM (1.4, 0.7, and 0.35 mg/kg, p.o. at 240 minutes) was found to be 0.442 ± 0.014, 0.501 ± 0.010, and 0.542 ± 0.013 ml, respectively. The LEM (1.4, 0.7, and 0.35 mg/kg, p.o. at 240 minutes) was found to be 0.491 ± 0.009, 0.542 ± 0.016, and 0.578 ± 0.013 ml, respectively. The LIM (1.4, 0.7, and 0.35 mg/kg, p.o. at 240 minutes) was found to be 0.547 ± 0.013, 0.582 ± 0.015, 0.635 ± 0.010 ml, respectively. The egg albumin is used as a phlogistic agent causes edema in rat hind paw. The egg albumin-induced hind paw edema designs are proper as screening agents for anti-inflammatory activity which are commonly used to evaluate the anti-oedemateous impact of the natural product [22,23]. Numerous inflammatory mediators like histamine, prostaglandins, and kinins including pro-inflammatory cytokinins have been proposed to perform a role in the mechanism of inflammation. Egg albumin significantly performed on the mast cells. Edema induced by it performs to be mediated by the release of histamine and serotonin [37,38]. The hind paw volume of egg albumin-induced paw edema of standard drug (Indomethacin, 10 mg/kg, p.o.), at 240 minutes was found to be significant as compared with *Ashodhita Manahshila* at 0.7 and 0.35 mg/kg in rats. But, after the treatment with ZM, SM, LEM, and LIM at 240 minutes were found to be significant as compared with control in egg albumin models (egg albumin-induced hind paw edema). The reduction of paw volume for untreated *Manahshila* indicates that the *Manahshila* has good contribution as an anti-inflammatory agent at a dose of 1.4 mg/kg, but the acute toxicity (trembling, piloerection, fore-limbs, and body extended, breathing complexity) was observed hence AM is not suitable for medicinal purposes. Therefore, it may be presumed that after *Shodhana,* anti-inflammatory activity of *Manahshila* became increased at the dose level of 1.4, 0.7, and 0.35 mg/kg, which indicates that purified *Manahshila* has excellent contribution as an anti-inflammatory agent and it's did not produced any such types of toxicity.

Conclusion

In the anti-inflammatory studies clearly revealed that the AM at a dose of 1.4 mg/kg p.o. inhibited the egg albumin-induced paw edema but it also produced acute toxicity in rats. The *Shodhita*

Manahshila (ZM, SM, LEM, and LIM) showed significant anti-inflammatory activity at all three dose levels in a dose-dependent manner as compared with control. The *Shodhita Manahshila* is also found safe for medicinal use. Thus, the study confirmed that the effect of *Shodhana* reduced the toxicity and facilitates the antioedematogenic effects on egg albumin-induced models even at a lower dose.

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ORIGINAL RESEARCH

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Ethnobotanical study of medicinal plants used as antimalarial and repellent by Sidama people of Hawassa Zuria district, Southern Ethiopia

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ABSTRACT

Background/Aim: More than 7,000 species of flowering plants are recorded in Ethiopia, of which only 200 species are recorded for malaria treatment. A large segment of the population in Ethiopia relies on traditional medicine to get a relief from various diseases. Malaria is the major cause of death in Southern Ethiopia. The main aim of the study was to assess the indigenous knowledge and to document antimalarial and repellent plants used by Sidama people of Hawassa Zuria district, Sidama zone, Southern Ethiopia.

Methods: A total of 150 informants (32 females and 118 males) were selected randomly to collect information on medicinal plants use from 10 kebeles. Out of these, 30 key informants were purposively selected based on the recommendation of the district office and elderly people. Ethnobotanical survey was conducted from January to February 2018. Ethnobotanical data were collected and analyzed through a semi-structured interview, field observation, Use value, preference ranking, and informant consensus factor.

Results: A total of 25 medicinal plants belonging to 24 genera and 19 families were recorded in the study area. Among the total traditional medicinal plants, 21 species were used as antimalarial and eight species were used as repellent. Out of the collected plant species, nine species (38%) were trees followed by shrubs (eight species, 33%). The highest informant consensus factor was scored for repellent (0.95). The most cited species were *Azadirachta indica* A. Juss. (UV = 0.50) followed by *Premna schimperi* Engl. (UV = 0.32) and *Dodonaea viscosa* subsp. *angustifoia* (UV = 0.19). The most preferred species by the informants were *Azadirachta indica* both as antimalarial and repellent plant.

Conclusion: The result of the current study showed the existence of indigenous knowledge of medicinal plants to treat malaria, as well as to repel mosquitoes and ticks in Hawassa Zuria district. Further research should be considered to discover effective antimalarial drugs and simple repellent products from the documented antimalarial plants through phytochemical and pharmacological studies.

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Introduction

Malaria is the major health problem in Africa, as well as in the world. Malaria transmission exists in 99 countries throughout the world and the greater burden of the disease is carried by African countries with the estimation of 212 million cases distributed in 45 countries [1]. Malaria is one of the fourth leading causes of death of children under the age of 5 years in developing countries [2]. Pregnant women and young children are most at risk of malaria. Malaria affects five times as many people as acquired immunodeficiency syndrome, leprosy, measles, and tuberculosis combined [3,4].

In Ethiopia, 75% of the landscape areas are below 2,000 m above sea level which is a malarious area and more than 54 million populations are at risk of malaria. It is becoming worse due to the development of Plasmodium parasite resistance against anti-malarial drugs, mosquito resistance to insecticides, increasing problems of toxicity to non-target

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organisms, lack of effective control measures, and no single effective method of malaria control. In Southern Ethiopia, approximately 65% of the population is living in malaria-endemic areas [5]. It is the primary causes of outpatient and inpatient consultation and hospital deaths in the SNNP region [6]. Small-scale studies documented 5.4% malaria parasite prevalence in Southern Nations, Nationalities, and Peoples Region in all age groups [7].

Effective and affordable vaccine drugs to control malaria are not available until these days. World Health Organization (WHO) recommends the following preventive and control strategies for malaria. These are Indoor residual spraying (IRS) with insecticides, insecticide-treated nets (ITNs), larval control, preventative chemotherapy, diagnosis and treatment [8]. The most widely used methods of management of malaria in Ethiopia are environmental management, use of insecticidal nets, and indoor spraying of insecticides [6]. Despite the current efforts to control malaria in Ethiopia, the situation has not improved mainly due to parasite resistance to the antimalarial drug, vector resistance to insecticides, low coverage of malaria preventative services, and poor access to health care [9].

The use of plant-derived compounds for mosquito control has been reported since 1933 [10]. Plants may be an alternative source of mosquito repellent agents because they constitute a rich source of bioactive chemicals [10]. Insect repellent plants are used to kill insects using different methods of application of medicinal plants. Plant repellents have an important place in protecting man from the bites of insect pests. Repellent plants can be used by smoking the plants and hanging on windows and doors, planting as a fence, spraying the leaves of the plant, and so on. The use of traditional repellents is widespread in different cultures and communities of Ethiopia. Different communities use different plants in various forms to protect themselves against mosquito and other insect bites [11].

Many of the major modern drugs such as quinine, salicylic acid, and artemisinin have been discovered from folk knowledge. For instance, Chinaberry (*Melia azedarach* L.) was recently investigated as a locally available, low cost, and sustainable insecticide that can aid in controlling malaria in Ethiopia [12]. Approximately 1,277 plant species and 160 families are used to treat malaria in the world, whereas a total of 200 different plant species from 71 families used for traditional malaria treatment were documented in different parts of Ethiopia [13]. Higher diversity of plants used to treat malaria was documented in the SNNP region, 94 plant species were reported in Southern Nation's National Peoples region [13]. A few ethnobotanical studies on antimalarial and repellent plants were conducted in Ethiopia [14–19].

However, the indigenous knowledge has not been systematically documented, especially there is no ethnobotanical study of antimalarial plants in Hawassa Zuria district. Documentation of traditional knowledge on medicinal plants is vital to maintain folk knowledge, conserve valuable plant resources, and rescue medicinal plants from loss. The reasons for loss of medicinal plants are deforestation, lack of written documents of medicinal plants, the death of elderly people without transferring the traditional skill to the member of the family, migration of people because of social problems, the influence of modern medicine, and exotic culture. Therefore, this study was conducted with the aim of documenting antimalarial and repellent plants and associated indigenous knowledge of Sidama people in Hawassa Zuria district. As a result to provide baseline data for further ethnopharmacological research of medicinal plants to treat malaria disease.

Research questions

1. What are the antimalarial and repellent plants in Hawassa Zuria district?
2. What type of diseases or insects are controlled by these plants?
3. Which part of the plant is used as a remedy?
4. What are the methods of preparation of the antimalarial plants?
5. What are the routes of administration of antimalarial plants?
6. What are the major threats to the loss of antimalarial and repellent plants?

Material and Methods

Description of the study area

The study area is located about 290 km South of Addis Ababa and 20 km South West of the regional capital Hawassa, in Sidama Zone of Southern Nations, Nationalities, and Peoples Region (SNNPR) of Ethiopia at 07°01'54"4 to 07°50'36"N latitude and 38°15'39" to 38°25'43"E longitude. The area size of the district is 22, 643 hectare and dry zone

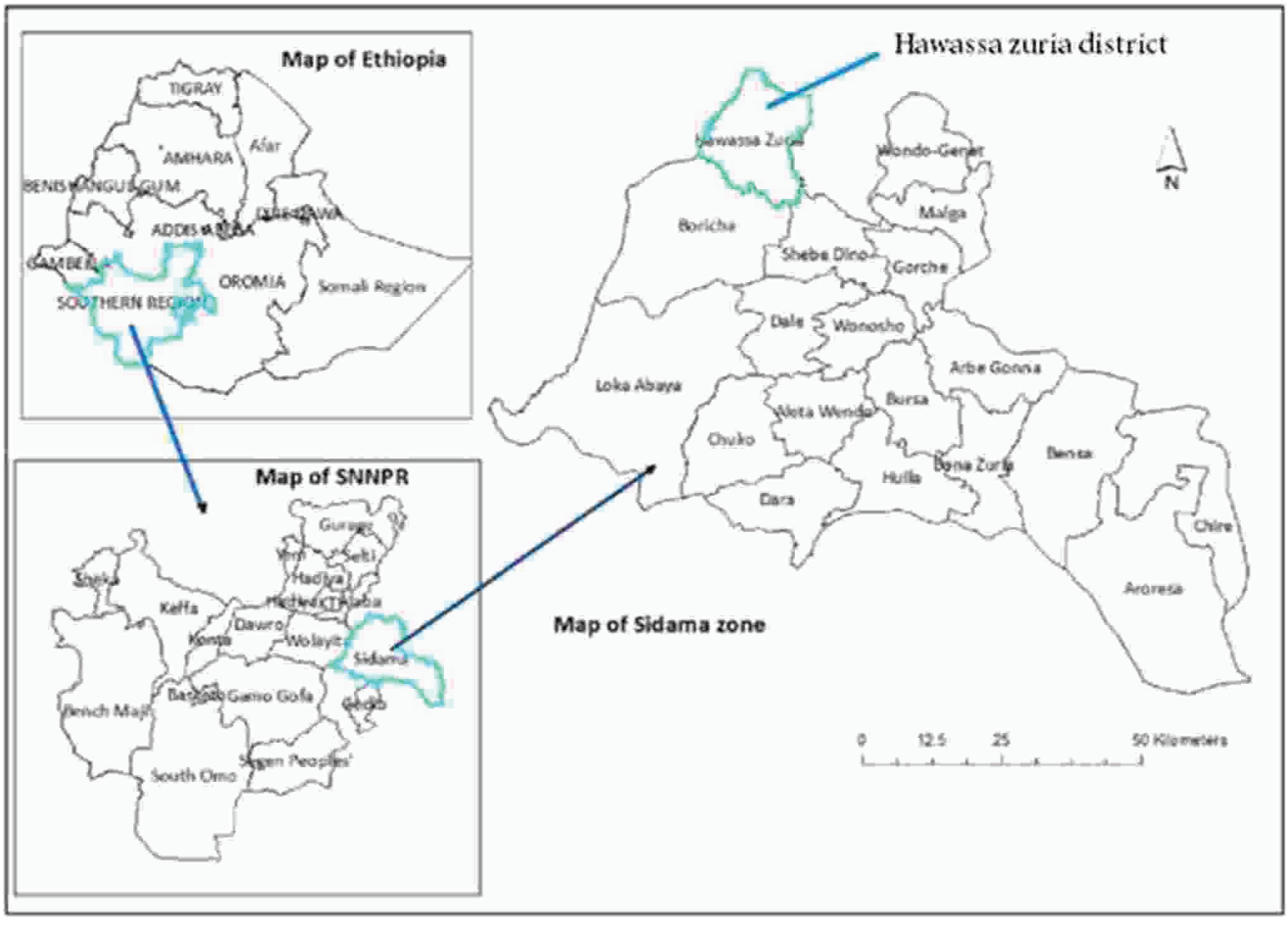

Figure 1. Map of Hawassa Zuria district, Sidama zone, Southern region, Ethiopia.

accounts 75% [20] (Fig. 1). Dore Bafeno is the administrative capital of Hawassa Zuria district.

Meteorological data recorded indicates that the rainy season spreads from March through September. The mean altitude of the district is 1,700 m above sea level. The annual rainfall ranges between 900 to 1,400 mm. Mean annual temperature ranges from 23°C to 27°C [20]. The current study area of Hawassa Zuria district falls within dry Woina Dega (Mid-altitude) category [21].

The Sidama ethnic group (19.38%) is the most predominant one in the SNNPR. The predominantly spoken language is also Sidamegna (18%) from the Cushitic linguistic family. The majority of people are followers of protestant religion (55.5%) [22]. The district has a total population of 124,472 of whom 62,774 are men and 61,698 women [22]. The total population density in the area is 465.5 people per square kilometer [23].

The most staple food of the local people in Southern Ethiopia is Enset [*Ensete ventricosum* (Welw.) Cheesman], which is locally called *wesse*, and is a unique food to Ethiopia. Hawassa Zuria district is well known by maize (*Zea maiz* Vell.) growing district. The other major growing crops are coffee (*Coffea arabica* L.), chat [*Catha edulis* (Vahl) Endl.], and sugarcane (*Saccharum officinarum* L.). In the study area, malaria is the primary public health problem and cause of the majority of people's death. The health station and clinics in Hawassa Zuria district are few in numbers.

Ethnobotanical methods

Ten kebeles were selected as study sites based on the attitude of the sites and vegetation cover. Thus, the study was carried out in 10 kebeles, namely Lebu Korem, Dore Bafeno, Jara Galalcha, Jara Qerara, Galo Argiso, Jara Damowa, Jara Dado, Doyo Otilcho, Tenkaka Ombulo, and Jara Hirnesa. Snowball sampling was used and the appointment was made prior to visit the informants. Semi-structured interview, guided field walk, and group discussion were used to collect Ethnobotanical data. The interviews were carried out in Sidamooafuu with a local translator

who had a good knowledge of the local community on the use of medicinal plants.

Ethnobotanical data collection

Informants and the administration were formally approached to get permission to do the research. A total of 150 (32 females and 118 males) informants were selected as recommended by the elders and local authorities in the district. Among the informants, 120 were general informants whereas 30 were key informants and 15 people were interviewed from each 10 kebeles, respectively. Ethnobotanical data were collected from January to February 2018. Ethnobotanical data were collected through an interview to gather the socio-economic status of the participants, usage and knowledge of antimalarial and insect repellent plants, name of antimalarial and repellent plants, plant parts used, method of application, growth habit, and routes of administration.

Plant specimen's collection and identification

Voucher specimens of the plants cited for their medicinal use were collected, numbered, pressed, and dried for identification. Plant identification was performed using the flora of Ethiopia and Eritrea book by comparison with authenticated specimens at Addis Ababa University, National Herbarium [24]. The identified plant species were further confirmed using the plant list website and finally deposited to the National Herbarium of Addis Ababa University.

Ethnobotanical data analysis

Descriptive statistical methods such as percentage and frequency were used to analyze and summarize the data on medicinal plants, use, and associated knowledge using Excel. Use value (UV) was calculated using the following standard formula: Use value: $UV = \sum U/n$, where U: number of use reports cited by each informant for a given plant species and n: total number of informants interviewed for a given plant [25]. The informant consensus factor was calculated using the formula: informant consensus factor (ICF) = nur-nt/nur-1, where ICF is informant consensus factor, nur is number of use citation, and nt is number of species used [26].

Medicinal plants were categorized into antimalarial and repellent groups. Repellent category consists of insects and ectoparasites such as housefly, honey bee, weevils, cockroach, corn worm, lice, and ticks, whereas antimalarial category includes malaria.

Preference ranking was conducted to rank the most frequently cited antimalarial plants by key informants following the approach of Martin [27], 10 key informants were invited to rank seven medicinal plant species that are used for the treatment of malaria. Values of 0 to 5 were used in this ranking (0 = not used, 1 = least used, 2 = less used, 3 = good, 4 = very good, and 5 = excellent) and the ranking were based on the informants perception.

Results

Sociodemographic characteristics of respondents

A total of 150 informants participated in this survey, 118 (78.6%) were males and 32 (21.4%) females**.** The majority of respondents were more than 50 years (42.4%) and 56 informants were with the age range between 36 and 50 (37.4%) and 30 informants were between 20 and 35 years (20%). The majority of informants were attended elementary school (48%), 76% were farmers, and 83% were followers of protestant religion (Table 1).

Antimalarial traditional knowledge

The number of antimalarial plants reported by males (23 species, 92%) was greater than female (13 species, 52%). The antimalarial plant species reported by the older (18 species, 72%) were greater than the younger (13 species, 52%) and adult informants (15 species, 60%). Regarding education status, illiterate people (20 species, 80%) had more traditional knowledge than the literate (8 species, 32%). This indicates that males, elder, and illiterate people are more knowledgable than the female, younger, and literate ones in treating malaria (Table 2).

Antimalarial and insect repellent plants

A total of 25 medicinal plants distributed in 22 genera and 18 families were documented. Among these, 21 species were used as antimalarial and eight plant species were recorded as repellent plants, namely *Croton macrostachyus* Hochst. ex Delile, *Datura stramonium* L., *Nicotiana tabacum* L., *Premna schimperi* Engl., *Schinus molle* L., *Azadirachta indica*, *Dodonaea viscosa* subsp. *angustifoia*, and Gagasa (Table 3). Among the reported plant species, Lamiaceae represented with four species (16%), followed by Solanaceae with three species (12%), and Euphorbiaceae with two species (8%). The rest Sapindaceae, Moringaceae, Asteraceae, Cucurbitaceae, and Amaryllidaceae were represented by one species (1, 4%) (Fig. 2).

Table 1. Sociodemographic details of the respondents in Hawassa Zuria district.

Social group	Variables	No. of informants (*n* = 150)	Percentage
Gender	Female	32	21.4
	Male	118	78.6
Age	Young (20–35)	30	20
	Adult (36–50)	56	37.4
	Older (>50)	64	42.6
Education	Illiterate	59	39.4
	Basic education	4	2.6
	Elementary (1–8)	72	48
	Secondary (9–12)	12	8
	Tertiary education (10+)	3	2
Occupation	Farmer	114	76
	Herbalist	20	13.4
	Birth attendants	7	4.6
	Merchant	5	3.4
	Student	4	2.6
Religion	Protestant	124	83
	Muslim	20	13
	None	6	4

Table 2. Traditional knowledge with respect to social groups.

Social group	Variables	No. of plants (*n* = 25)	Percentage
Gender	Female	13	52
	Male	23	92
Age	Young (20–35)	13	52
	Adult (36–50)	15	60
	Older (>50)	18	72
Education	Illiterate	20	80
	Literate	8	32

Seven plant species were recorded as repellent plants, namely: *Croton macrostachyus* Hochst. ex Delile, *Datura stramonium* L., *Nicotiana tabacum* L., *Premna schimperi* Engl., *Schinus molle* L., *Azadirachta indica*, and *Dodonaea viscosa* subsp. *angustifoia* The majority of repellent plants were used to control ectoparasites such as ticks and lice. About 41% of repellent plants were used as ticks control, whereas the rest controls lice (16%), corn worm, weevils, cockroach, housefly and bee (8% each), and mosquito (3%) (Fig. 3).

Growth habits, forms of plants, and plant parts used

The commonly used growth habits reported by the respondents were trees (nine species, 38%), followed by shrubs (eight species, 33%), herbs (five species, 21%), and climbers (two species, 8%) respectively (Fig. 4). Most of the antimalarial and repellent plants were collected from the wild forest, home garden, and roadsides. Most of the remedies (79%) were prepared from freshly harvested plants and 21% were prepared in dry form. The informants reported that plant parts were collected whenever needed and there is no specific time of collection. The majority of the remedies were prepared from leaf (75%), followed by stem and fruit (8% each). The rest were prepared from shoot tip, bulb, and root (3% each), respectively (Fig. 5).

Remedy preparation and route of administration

The majority of the remedy was prepared by crushing (21%), followed by rubbing (12%) and directly by eating (10%). The rest were prepared in liquid form, by burning and boiling (7%) each respectively. Some informants prepared the remedy by chewing, spraying, and mixed with other foods (5%). The remaining remedy was prepared from powdering (2%) the plant parts (Fig. 6). Some of

Table 3. Antimalarial and repellent medicinal plants of Hawassa Zuria district, Southern Ethiopia.

Family	Scientific name	Local name	V. No.	Ha	PPU	FOP	MOP	ROA	Use	UV	Literature
Amaryllidaceae	*Allium sativum* L.	Netch shinkurt (Amh)	BN077	H	Bu	Fresh	Crushing	Oral	Malaria	0.11	[14,28–32]
Anacardiaceae	*Schinus molle* L.	Qundo berbere(Amh)	ET054	T	L	Fresh	Put the leaf onto meat	External	Housefly	0.01	[33]
Asteraceae	*Vernonia amygdalina* Delile	Girawa (Amh)	BN076	Sh	Sht	Fresh	Crushing	Oral	Malaria	0.06	[14,28–31,34–38]
Cucurbitaceae	*Peponium vogelii* (Hook.f) Engl.	Surupa (Sd)	ET014	Cl	Fr	Fresh	Eating	Oral	Malaria	0.14	[14]
Caricaceae	*Carica papaya* L.	Papaya (Amh)	BN082	T	Fr, L	Fresh	Crushing	Oral	Malaria	0.03	[28,31,32,37–41]
Euphorbiaceae	*Euphorbia abyssinica* J.F. Gmel	Qulqual (Amh)	ET004	T	L, Lx	Fresh	Chewing	Oral	Malaria	0.01	[28]
Euphorbiaceae	*Croton macrostachyus* Hochst. ex Delile	Masincho (Sd)	ET022	T	L	Dry	Burning , smelling	dermal	Malaria, Ticks	0.02	[14,28–30]
Lamiaceae	*Rotheca myricoides* (Hochst.) Steane & Mabb.	Madisisa (Sd)	BN025	Sh	St	Fresh	Cutting	Oral	Malaria	0.01	[14]
Lamiaceae	*Ajuga integrifolia* Buch.-Ham.	Anamuro(Sd)	BN031	H	L	Fresh	Crushing	Oral	Malaria	0.01	[14]
Lamiaceae	*Premna schimperi* Engl.	Udo (Sd)	BN063	T	L	Fresh	Grinding and rubbing	External	Tick, bee	0.33	[14,28]
Lamiaceae	*Ocimum gratissimum L.*	Angabisha (Sd)	ET015	Sh	L	Fresh	Crushing	Oral	Malaria	0.01	[14]
Meliaceae	*Azadirachta indica* A. Juss.	Mimi (Sd)	ET005	T	L	Dry	Crushing	Oral	Malaria, Lice, weevil	0.50	[14,28,31]
Moringaceae	*Moringa stenopetala* (Baker f.) Cufod.	Haleqo (Sd)	ET009	T	L	Dry	Grinding and rubbing	Oral	Malaria	0.14	[14,34,37,40,42]
Myrtaceae	*Eucalyptus globulus* Labill.	Netch bahir zaf (Amh)	ET023	T	L	Fresh	Liquid form	Oral	Malaria	0.05	[14,31]
Papaveraceae	*Argemone mexicana* L.	Kokole (Sd)	ET008	H	St, L	Fresh	Eating	Oral	Malaria	0.03	[14]
Podocarpaceae	*Afrocarpus falcatus* (Thunb.)	Dagucho (Sd)	BN056	T	L	Fresh	Crushing	Oral	Malaria	0.01	[34]
Ranunnculaceae	*Nigella sativa* L.	Tikur azemud (Amh)	BN078	H	Fr	Fresh	Mixed with garlic	Oral	Malaria	0.01	[43]
Rhamnaceae	*Rhamnus prinoides L'* Her.	Gesho (Amh)	ET002	Sh	L	Fresh	Mixed with honey	Oral	Malaria	0.02	[28]
Sapindaceae	*Dodonaea viscose subsp. angustifoia* (L.f.) J.G.West	Ittancha (Sd)	ET011	Sh	L	Dry	Crushing	Oral	Malaria, ticks	0.19	[28,29]

Continued

Family	Scientific name	Local name	V. No.	Ha	PPU	FOP	MOP	ROA	Use	UV	Literature
Solanaceae	*Solanum americanum* Mill.	Tunaye (Sd)	ET003	H	L	Fresh	Boiling and eating	Oral	Malaria	0.03	[44]
Solanaceae	*Nicotiana tabacum* L.	Arado (Sd)	BN060	Sh	L	Dry fresh	Grinding, rubbing, boiling	External	Ticks, Corn worm	0.03	[31,45]
Solanaceae	*Datura stramonium* L.	Banje (Sd)	ET020	Sh	L	Fresh	Liquid form	Oral	Malaria Ticks	0.02	[46]
Unknown	Unknown	Gagasa (Sd)	BN024	un	L	Dry	Burning	External	Coackroach, Lice	0.01	No report
Vitaceae	*Ampelocissus bombycina* (Baker) Planch.	Molama (Sd)	ET049	Cl	R	Dry	Crushing	Oral	Malaria	0.01	No report
Xanthorrhoeaceae	*Aloe adigratana* Reynolds.	Eret (Sd)	ET028	Sh	L, St	Fresh	Liquid form	Oral	Malaria	0.16	No report

V. No. = Voucher number; Ha = Habitat, T = Tree, Sh = shrub, H = Herb, Cl = Climber, PPU: Plant part used, St = Stem, L = Leaf, R = Root, Fr = Fruit, Lx = Latex, Sht = Shoot tip, Bu = Bulb, MOP = Methods of preparation, ROA = Routes of administration ; FOP = Forms of plants, UV = Use value.

the ingredients mixed during the antimalarial remedy preparation are milk, honey, water, and other medicinal plant species. This ingredient makes the preparation of remedy to be very strong and effective for malaria treatment.

The majority of the antimalarial medicinal plants were administered orally (64%) and followed by dermal (3%). The repellent plants were mainly applied externally in smoke form. Some people just use the repellent plants by hanging around their bedroom, door, and windows.

Informant consensus factor

A total of 25 species were identified to treat malaria, repel insects and ectoparasite. The categories with the highest ICF values were repellent (0.95), followed by antimalarial (0.91) (Table 4). A high ICF value (0.95) indicates the informant's uses relatively few taxa to manage the specific disease. According to the informant consensus data analysis, *Azadirachta indica* with citation by 75 informants (50%) ranked first followed by *Dodonaea viscosa* subsp. *angustifoia* (29 informants, 19%) and *Aloe adigratana* Reynolds. (24 informants, 16%) for antimalarial disease category. For repellent category group, *Azadirachta indica* scored also the first rank with 75 informants (50%) citation, followed by *Premna schimperi* (49 informants, 33%) and *Dodonaea viscosa* subsp. *angustifoia* (29 informants, 19%).

Preference ranking of antimalarial medicinal plants

Ten key informants were asked to rank seven selected medicinal plant species used against malaria disease. The result showed that *Azadirachta indica* was the most preferred and followed by *Aloe adigratana*, *Moringa stenopetala* (Baker f.) Cufod., *Peponium vogelii* (Hook.f) Engl.), and *Allium sativum* L. in the preference ranking conducted by key informants (Table 5). This is due to the preferred plant species were strong enough to treat malaria disease and also easily availability of the plant from the home garden and roadside.

Discussion

Plants play an important role in every aspect of our lives and without them, life is not possible. One of their major roles is as a medicinal plant to treat different ailments and also as an alternative source of insect repellent agents. Repellent plants have an important place in protecting human from the bites of insect pests and livestock from ectoparasites

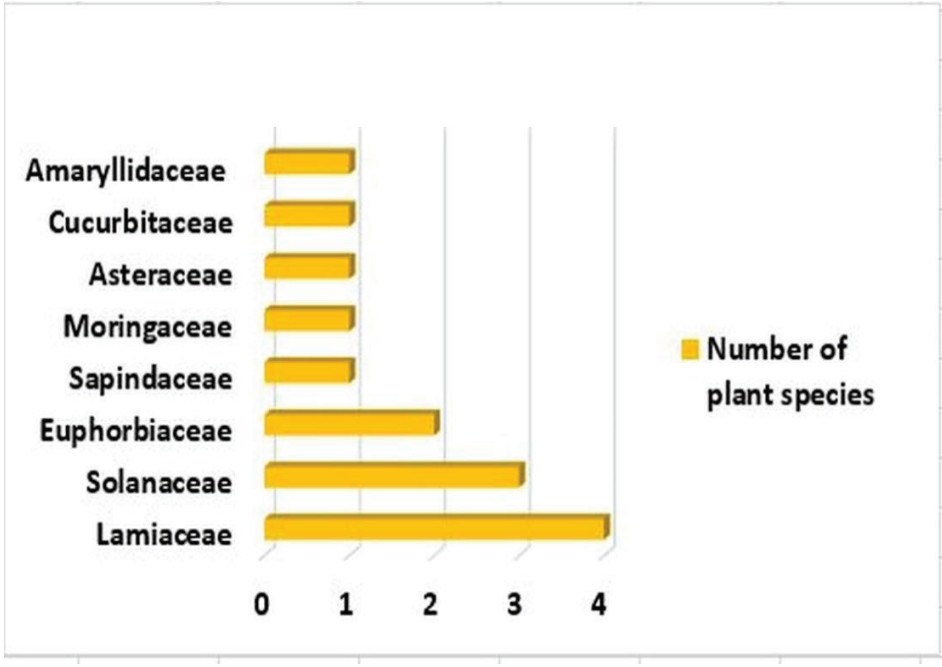

Figure 2. Distribution of plants families in the study area.

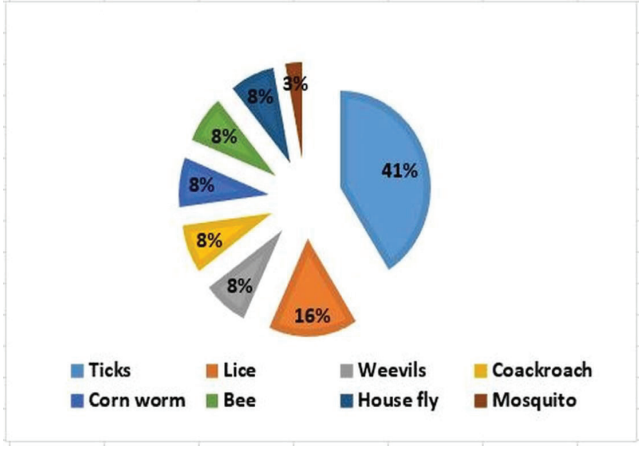

Figure 3. Types of insects and ectoparasites.

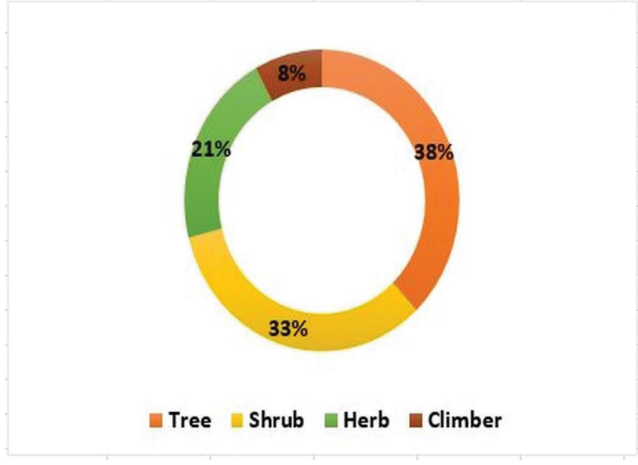

Figure 4. Plant growth habits.

such as tick. More than half of the population in Ethiopia relies on medicinal plants. This is due to the high cost and unavailability of modern drugs, as well as faith on the potential of traditional medicines. Malaria is one of the major health problems in the study area and there is no vaccine for malaria until today.

In the present study, a total of 25 medicinal plants were documented. The majority of these medicinal plants were reported for use in the treatment of malaria whereas the rest were used as repellent plants to drive away some insects and ectoparasites. Thus, this indicates the existence of medicinal plants used as antimalarial and repellent plants for the control of malaria disease in Hawassa Zuria district.

Regarding the sociodemography details of the respondents, the majority of the respondents were male, attend elementary school, farmers, and followers of the Protestant religion. Previous ethnobotanical studies [47–50] reports similar findings. Regarding the traditional knowledge of informants, male, older, and illiterate people were more knowledgeable than female, younger, and literate people, respectively. This is due to high secrecy of traditional knowledge by older peoples, the transferring of knowledge to the first son of family member than daughter, modernization and exotic culture influence, unwillingness of the young generation to be a traditional practitioner like their ancestors. In the current study, elder people were

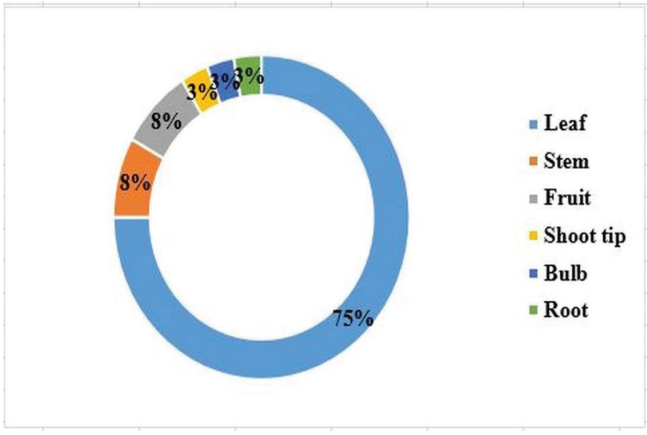

Figure 5. Plant parts used in the study area.

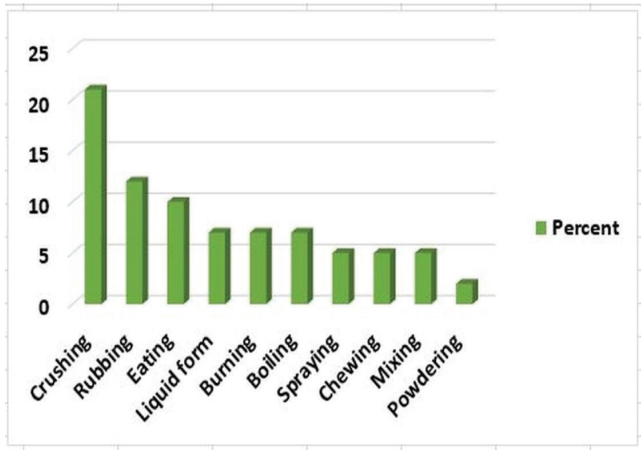

Figure 6. Methods of preparation of antimalarial and repellent plants.

the highest respondents than the young. This indicates the older people have experience in the practice of traditional medicine and responsible for transferring the knowledge to the younger generation. However, this indigenous knowledge is not shared among all other communities equally. Therefore, the motivation of both the elder and young generation to share and accept the indigenous knowledge is the key to fill the gap beside the documentation of the indigenous knowledge for further research.

In the current study, *Nigella sativa L., Schinus molle L., Euphorbia abyssinica* J.F. Gmel*, Rhamnus prinoides L'* Herit., *Solanum americanum* Mill., and *Ampelocissus bombycina* (Baker) Planch were recorded as antimalarial and repellent plants. It is important to test the antimalarial activity of these medicinal plants to find out their effectiveness for future use. *Carica papaya* L. were mostly reported as antimalarial plant species in Awash Fentale district [49], Sasiga district [47], and Jimma zone [19]. *Allium sativum* [51–53] and *Argemone mexicana* [54] were mentioned in previous studies as antimalarial plants. The current study identified *Croton macrostachyus*, *Datura stramonium*, *Nicotiana tabacum* L., *Premna schimperi*, and *Dodonaea viscosa* subsp. *angustifoia* species for the control of ticks. Repellent activities of these plants should be extracted and tested for the management of ticks. In previous ethnobotanical studies, *Croton macrostachyus* [19,48] and *Nicotiana tabacum L.* [52] were documented as an antimalarial and repellent plant.

Most of the antimalarial and repellent plants in the study area were collected from the wild forest, home garden, and roadsides. Thus, the majority of the antimalarial plants were harvested from the home garden and roadsides. This result is similar

Table 4. ICF values by categories for treating malaria and other ectoparasites.

Category	List of plant species used and number of citations in the bracket	Total number of		ICF
		Species	Use citations	
Antimalarial	*Allium sativum* L. (17), *Vernonia amygdalina* Delile (9), *Peponium vogelii* (Hook.f) Engl. (21), *Carica papaya* L. (4), *Euphorbia abyssinica* J.F.Gmel (1), Croton *macrostachyus* Hochst. ex Delile (3), *Rotheca myricoides* (Hochst.) Steane & Mabb. (1), *Ajuga integrifolia* Buch.-Ham. (2), *Ocimum gratissimum* L.(1), *Azadirachta indica* A. Juss (75), *Moringa stenopetala* (Bak.f) Cufod. (21), *Eucalyptus globules* Labill (8), *Argemone Mexicana* L. (4), *Afrocarpus falcatus* (Thunb.) (1), *Nigella sativa* L. (1), *Rhamnus prinoides L'* Herit. (3), *Dodonaea viscosa* subsp. angustifoia (29), *Solanum americanum* Mill..(4), *Datura stramonium* L. (3), *Ampelocissus bombycina* (Baker) Planch. (1), *Aloe adigratana* Reynolds. (24)	21	233	0.91
Repellent	*Schinus molle* L. (2), *Croton macrostachyus* Hochst. ex Delile.(3), *Premna schimperi* Engl.(49), *Azadirachta indica* A. Juss (75), *Dodonaea viscosa* subsp. *angustifoia* (29), *Nicotiana tabacum* L. (4), "Gagasa" (2), *Datura stramonium* L. (3)	8	167	0.95

Table 5. Preference ranking of medicinal plants used against malaria disease.

Antimalarial plants	Informants (I1–I10)										Total score	Rank
	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10		
Azadirachta indica A. Juss	5	5	4	4	5	3	5	5	4	5	45	1st
Aloe adigratana Reynolds	4	4	3	3	5	3	4	4	4	4	39	2nd
Moringa stenopetala (Baker f.) Cufod.	4	4	3	3	3	4	3	4	4	3	35	3rd
Peponium vogelii (Hook.f) Engl.	3	4	4.5	4	4	3	4	3	3	2	33	4th
Allium sativum L.	2	2	3	3	3	3	2	3	2	2	24	5th
Vernonia amygdalina Delile	2	2	1.5	1	1	2	3	2	1	1	16	6th
Eucalyptus globules Labill.	2	1	1.5	2	1	2	2	1	1	1	14	7th

Note: I1–I10 represent informants 1–10.

with other studies conducted in Ethiopia, as well as other countries of the world. Around 49.0% of the antimalarial plants were harvested from the forest, whereas 39.0% were from roadsides in Boricha district [14].

In Hawassa Zuria district, the major families that contributed more medicinal species were Lamiaceae (four species), followed by Solanaceae (three species) and Euphorbiaceae (two species). This could be an indication for considerable diversity of plant species. Ethnobotanical research reports have also shown that these families had high domination in Ethiopia [8,14], as well as in other countries [55–58].

Trees (38%) were the most widely used growth forms from which Sidama people of Hawassa Zuria district prepare herbal remedies. This is in agreement with the studies reported 59% of the identified populations in Nigeria were trees [59]. 50% of the medicinal plants were trees in Cameroon [60]. However, other findings [8] indicated herbs were the most frequently used plant categories.

The plant parts used to treat malaria disease varied from species to species. Leaf was reported as the dominant plant part for antimalarial remedy preparation in the study area. Similar findings indicated leaf as a major dominant plant part in Ethiopia [14,49,57,61] for herbal medicine preparation. The most frequently used plant part was also leaf in Kenya [57] and Ghana [62]. The preference of leaves was due to its easy availability and simplicity in remedy preparation. In addition, the less effect to the whole plants makes leaf as the most preferred one than other plant parts. These uses of plant parts are also important for the conservation of medicinal plants as it cannot cause the death of the whole plant. Stems and fruits were the other used plant parts next to leaves. However, root was mentioned as the most frequently used plant part in Southern Ethiopia [63].

Antimalarial and repellent medicinal plants in the study area were prepared in different ways. The majority of the remedies in the study area were prepared by crushing followed by rubbing the plant parts. Ethnobotanical research survey conducted elsewhere in Kenya showed the majority of the respondents prepared the remedy by decoction [64]. Other methods of remedy preparation used were burning, smelling, rubbing, and boiling. Burning to generate smoke was reported as the major methods of remedy preparation to drive away insects [61]. Boiling was also reported as the most used methods of remedy preparation in Ghana [62]. Smoke by burning was reported as a major application of repellent plants in Tigray [17]. Decoction cited as the major mode of preparation in Togo [65]. Burning and smoldering were reported as the commonly used methods of remedy preparation [48] Decoction mentioned as the major methods of preparation in Guji zone [66].

The malaria disease was mainly treated by taking the prepared remedies orally, whereas repellent plants were applied externally. This result was similar to the findings of previous studies that revealed oral as the major route of administration in Guji zone [66] and Bennin [54]. Higher number of respondents uses repellent plants to control ectoparasites such as ticks and lice. Research findings in Kenya [67] showed repellent plant species had the highest repellency for ticks.

The current study revealed that *Azadirachta indica* as the most preferable medicinal plants for the treatment of malaria, followed by *Premna schimperi* and *Dodonaea viscosa* subsp. *angustifoia.* Similar findings reported that *Azadirachta indica* as the dominant antimalarial plants in Somali region [53] and Nigeria [52]. Other findings also reported protection against mosquito bites by these species [28,68]. Among the various species of the genus Premna, *Premna angolensis* (Lamiaceae) has been

reported to have repellency potential [69]. Thus, *Premna schimperi* should be investigated for its antimalarial and repellency activity. Antimalarial plants have promising therapeutic potential in different African countries. Previous findings reported that promising candidates have been identified from the antimalarial plant [70–73]. Therefore, further research should be considered for the current documented antimalarial plants to identify the therapeutic potential of promising antimalarial plants.

Among the documented antimalarial and repellent medicinal plants in Hawassa Zuria district, 22 species were mentioned in previous ethnobotanical studies conducted in Southern Ethiopia [29–32,34,37–43,45], North West Ethiopia in Shinasha [33], Chelya Woreda [46] and [44]. Whereas three species were mentioned for the first time as antimalarial and repellent plant in Hawasssa Zuria district. The two plants were *Ampelocissus bombycina* and *Aloe adigratana*, for treating malaria disease. The third species were not identified scientifically yet but it is known by the local name called "Gagasa" and used for cockroach and lice control by Sidama people in Hawassa Zuria district. Therefore, further efficacy test should be considered for those new candidate species that were documented for the first time in the current study area.

Conclusion

The present study findings indicate that the Sidama people of Hawassa Zuria district have rich traditional knowledge of medicinal plants to treat malaria and repel insects pests. However, the knowledge is mainly elderly people-centered. The young generation have/had little knowledge regarding traditional knowledge. Thus, this might lead to the loss of indigenous knowledge and medicinal plants from the area. Usage of medicinal plants other than their medicinal value leads to the overexploitation of medicinal plants. In Hawassa Zuria district, the major threats for the loss of antimalarial and repellent plants and associated knowledge were deforestation, agriculture expansion, urbanization, and firewood collection. The other reason for the loss of indigenous knowledge is the secrecy of elder people and their willingness to transfer their knowledge only to the first son of the family member and this might lead to the death of elder people without transferring the knowledge. Therefore, awareness of the community concerning traditional knowledge and creating medicinal plants conservation strategy is vital in order to rescue medicinal plant threats. Beside the antimalarial plants, repellent plants are also promising for tick controls in this study. Therefore, repellency effectiveness of plants against tick should be further tested for ticks management. Further research is needed to identify the therapeutic potential of antimalarial and repellent plants.

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MINI REVIEW

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Drugs and dietary supplements with unproven effects in research and practice

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ABSTRACT

It is evident for a reviewer of scientific literature that the quality of argumentation in some areas of medical research has deteriorated during the last decades. Publication series of questionable reliability have been continued without making references to the published criticism. Another tendency is that drugs without proven efficiency are advertised, corresponding products patented and marketed as evidence-based medications. Professional publications are required to register drugs and dietary supplements to obtain permissions for the practical use; and such papers appeared, sometimes being of questionable reliability. Several examples are discussed in this review; when substances without proven effects were introduced into practice being supported by publications of questionable reliability. Some of the topics are not entirely clear, and the arguments provided here can induce a constructive discussion.

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Introduction

The profligate prescribing has brought a hidden epidemic of side effects and no benefit to many patients [1]. It is evident for a reviewer of medical literature that the quality of argumentation has decreased since the last decades. Dubious publication series have been continued without references to published comments; several examples are discussed here. Another tendency is that drugs and dietary supplements without proven efficiency are advertised as evidence-based medications. Professional publications are needed to register drugs and treatments for permissions of practical use. It is, therefore, not surprising that papers of questionable reliability appeared in large numbers. Artificial scientific concepts have been construed for that purpose or existing ones used inadequately [2]. In this connection, the problem of extensive citation of papers published in unknown and local journals should be mentioned. The peer-reviewing in such journals is not always adequate, while papers of questionable quality, with insufficient evidence and argumentation, overt or hidden conflicts of interest are sometimes published. Paid open access publication is a separate topic; some conflicted researchers use their subsidiary earnings to publish biased papers in such editions. Although the international literature is cited in this review, it is focused on the Russian studies, as well as some aspects of drug marketing and governmental regulation in the Russian Federation. Some dietary supplements or drugs discussed here are registered as such only in the countries of the former Soviet Union (SU). However, the tendencies mentioned above seem to be global so that the conclusions of this review can be generalized to some extent. In particular, certain medications used in the folk or traditional medicine of some countries lack scientific corroboration, which might be a topic for a separate review. The conclusions are partly based on theoretic considerations. When the literature is so abundant, while it is difficult to distinguish between reliable and unreliable reports, theoretic considerations gain in importance. The placebo therapy can be beneficial and ethically justifiable, but it is not a sufficient reason to publish biased information in support of the placebo marketing. Note that some substances without proven effects are quite expensive. With pharmaceutical costs increasing faster than most other health care expenditures, studies must meet

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the needs of evidence-based treatments and not just the needs of the manufacturers [3]. Practical recommendations must be based on the research of high quality shielded from conflicts of interest. Only such research should be included in reviews and meta-analyses.

Testing of Anti-Atherogenic Drugs in Cell Cultures

Strategies to treat atherosclerosis should take its multifactorial etiology into account [3]. Atherogenesis involves many cell types interacting with each other and with the extracellular matrix. Therefore, results obtained in studies on a single cell type should be considered with caution when extrapolated to the whole organism. The large research series has become known to the international scientific community in the mid-1980s [4], being continued until today [5–25]. Cell cultures were used to evaluate the capacity of various substances to increase or decrease the cholesterol accumulation in the cytoplasm, which was interpreted as pro- or antiatherogenic effects. After 1 day's incubation with diluted serum from atherosclerosis patients, the amount of cholesterol in the cytoplasm of cultured cells reportedly increased two- to five-fold. Low-density lipoproteins (LDL) from atherosclerosis patients induced a two- to four-fold increase in the intracellular cholesterol. The incubation with a serum or LDL from controls did not induce any intracellular cholesterol accumulation [9,10]. According to a personal communication from Denis Aksenov at the 77th EAS Congress in Istanbul [11], the "cell cultures" did not grow. Accordingly, it would be correct to designate these cells, persisting *in vitro* several days or weeks, not as cultures but as incubated cells.

Along with drugs and other substances, the same *in vitro* method was applied for the assessment of sex hormones: reportedly, both testosterone and estrogens diminished the accumulation of cholesterol in the cytoplasm along with the reduction of ^{3}H-thymidine incorporation as an index of cell proliferation [12]. Notably, excessive accumulation of lipids in the cytoplasm (fatty change) is a degeneration that should hardly be expected to come along with enhanced proliferation. Surprisingly, a testosterone analog dihydrotestosterone was reported to exert an opposite effect: the enhanced cholesterol accumulation and DNA synthesis by cell cultures. Estrogens suppressed the intracellular cholesterol accumulation. Conversely, androgens elevated the accumulation and incorporation of ^{3}H-thymidine [12]. In a more recent report, both estrogens and androgens enhanced the cholesterol accumulation [11]. Later on, estradiol, testosterone, and dihydrotestosterone in physiological concentrations were reported to decrease intracellular cholesterol [8]. The above controversies question the reliability of reports by this research team.

The following botanical substances have been reported and patented by the same research team as anti-atherogenic agents: licorice root, onion, black elderberry, marigold blossom, and violet herb [6,13–15,20–23]. Anti-atherogenic properties were reported for certain mushrooms [15], fish products [24], pine needles [25], "prolonged and pronounced antiatherosclerotic effect of wheat seedings," etc. [7]. In the author's opinion, these results are questionable. Moreover, drug dosages have been calculated on the basis of the cell culture experiments: "To decrease atherogenic potential of serum and to maintain it at a low level, verapamil should be administered at a dose of 40 mg five times daily; three with a 4- to 5-hour interval between doses" [5]. Such recommendations might be unsafe if taken seriously by clinicians; more details and references are in Jargin [2].

In a living organism, the relationship between the cholesterol uptake by cells and atherogenesis is inverse rather than direct. The blood level of LDL depends on the function of LDL-receptors. For example, in familial hypercholesterolemia, a dysfunction of lipoprotein receptors results in advancing atherosclerosis. In cultures, many cells rely on receptors for the supply of cholesterol [26]. Lipoprotein receptors both of the classical and of scavenger types were found on macrophages and smooth muscle cells [27–30], i.e., cell types used in the research series under discussion [4–25]. The role of scavenger receptors in the uptake from the blood of modified LDL is analogous to that of classical receptors for native LDL [30,31]. A pharmacological agent, lowering the blood atherogenicity directly as per Sobenin et al. [6], or by means of receptors, suppressing the cholesterol uptake by cell cultures, i.e., by entire cell populations, would elevate the blood cholesterol level *in vivo*. By analogy with familial hypercholesterolemia, this would contribute to atherosclerosis. Lipids would be deposited primarily in sites with the injured endothelial lining, or in vulnerable atherosclerotic plaques—so as it occurs in advancing atherosclerosis. This mechanism has been disregarded by the authors

[4–25]: the agents having an "anti-atherogenic" effect *in vitro* would lower the cholesterol clearance from blood; thus, elevating the blood cholesterol. Atherosclerosis is a focal disease, affecting especially injured sites of the vascular lining, which would be favored by hypercholesterolemia. So, the supposedly anti-atherogenic preparations, proposed on the basis of the cell culture experiments [13–15, 20–23], would have a pro-atherogenic effect, if any. No other studies are known, where atherogenic or anti-atherogenic effects would be evaluated in cell cultures and clinical recommendations formulated on the basis of such research. Other studies of cultured or incubated cells included neither measurements of serum atherogenicity nor drug testing [32–34]. The doubtful information has been published in diverse pharmacological and clinical journals. The above criticism has been published repeatedly, e.g., [2,35,36] and presented at conferences [37] but never cited by the authors [4–25] in spite of personal communications. Considering the above, at least some of the papers [4–25] should be formally retracted.

Carnosine and Antioxidants

Carnosine (β-alanyl-L-histidine), an endogenously synthesized dipeptide found in muscular and other tissues, was reported to possess antioxidant properties, i.e., to counteract damage incurred by reactive oxygen species (ROS) and peroxynitrite (reactive nitrogen species) [38–43]. Furthermore, a mechanism of detoxification from aldehydes, accumulating in inflammation, ischemia, and other pathological conditions, was supposed to be conjugation with carnosine [44–47]. Data on the use of carnosine in various conditions are discussed here. Special attention is given to neurologic and mental diseases, where favorable effects might be caused by a nutritive value of the substance, especially in conditions of malnutrition and protein deficiency. The following fallacy can be encountered in publications about carnosine and other normal tissue constituents. First, an important biochemical role is discussed, which is obvious for a substance normally participating in the metabolism. After that, the favorable effects of intake of this substance are postulated, although it is unknown, whether there is a deficiency or not. Certain pharmaceuticals are both expensive and unnecessary, having no advantage over a diet modification if supplementation is necessary indeed. A proper diet containing enough animal products would probably suffice.

Among other proposed applications, carnosine eye drops have been recommended for the treatment and prevention of cataracts [42,48]. The topical application of carnosine as eye drops does not result in penetration into the eye. Therefore, a histidine-containing dipeptide N-acetylcarnosine (NAC) has been used as a vehicle penetrating through the cornea. The metabolism of NAC within the eye produces L-carnosine, supposed to be the active substance [49]. Physiologically, it is hard to comprehend how carnosine brings about a reversal of cataracts that are in fact consequences of conformational changes and aggregation of proteins. According to a recent Cochrane Review, there is no convincing evidence that NAC reverses cataract, nor prevents progression of cataract defined as a change in cataract appearance either for the better or for the worse [49].

A deficiency seems to be improbable for a substance that is present in food and produced by the body. The primary source of carnosine is the dietary intake of meat and fish [50]. The carnosine level in tissue fluids depends on the diet being lower in vegetarians and vegans [38,41,51]. If the concentration of carnosine is essential, e.g., for the lens transparency, the incidence of cataracts would be higher among vegetarians than in the general population. However, vegetarians tend to have a lower cataract incidence than people consuming animal products. A significant relationship was reported between the risk of cataracts and the diet, with a progressive risk decline from people consuming much meat to those consuming less meat, fish consumers, vegetarians, and vegans [52]. Admittedly, the data are not uniform [53], being cofounded by the exposure to sunlight, the race, etc. Furthermore, it has been proposed that carnosine is an agent counteracting aging and having favorable cardiovascular effects [40,54,55]. However, vegetarians tend to have lower rates of ischemic heart disease and arterial hypertension. The average incidence of cancer in vegetarians tends to be slightly lower than in the general population, their life duration being averagely higher, although they receive no carnosine with the diet [56,57]. The use of carnosine in the sports nutrition and reported favorable effects in diabetes mellitus [58] are explainable as this oligopeptide is a nutrient not containing carbohydrates. Favorable effects of carnosine, taurine, and other meat-derived substances with nutritive value in people with dementia and mental disorders [59] can be regarded as indicators of malnutrition or

protein deficiency, e.g., in some homes for the aged and psychiatric facilities [60].

As for the supposed anti-oxidative effect, it should be taken into account that ROS are generated normally in the process of tissue respiration and may have either harmful or favorable effects [61]. The latter is true for carnosine [62]. The redox status is kept in a dynamic balance under the impact of various internal and external factors [63–65]. There is an opinion that exogenous anti-oxidative agents exert no substantial favorable effects in many conditions when they are supposed to be indicated [63]. Some papers about antioxidants discuss vitamins and other agents with multifaceted mechanisms of action [66–68]. It is often unclear which antioxidants (if any) should be taken, how much, and how long [61,69,70]. The intake of antioxidant supplements would only make sense in the case of a deficiency, the latter being difficult to prove. Apparently, the best way to minimize a redox imbalance is a well-balanced diet [71].

Last but not least, the peribulbar injections of carcinine (the analog of carnosine) as a supposed antioxidant [72] are precarious because of the risk of complications [73]. The same is true for other meat-derived substances such as taurine (Taufon) applied for the treatment of age-related and inflammatory ophthalmic conditions [74,75]. Hematomas were seen to complicate such injections. Besides, various antioxidants for peribulbar injections have been patented [76–78]. More details are in the study of Jargin [60]. Peribulbar injections of Mildronate are discussed in the next section.

Meldonium (Mildronate)

Meldonium (Md) [3-(2,2,2-trimethylhydrazinium) propionate dihydrate], named also Mildronate, is a pharmaceutical competitively inhibiting gamma-butyrobetaine hydroxylase, the enzyme participating in the biosynthesis of carnitine [79]. In a series of studies from the former SU, the efficiency of Md was reported in cardiac insufficiency, myocardial infarction, and stroke. Considering a potential reduction in the availability of Adenosine triphosphate (ATP) as the energy carrier and other reasons discussed below, Md may contribute to a decline in the cardiac function in some patients with myocardial infarction and heart failure. The problem should be seen within the scope of placebo marketing under the guise of evidence-based medications. Note that placebo with potential adverse effects is named pseudo-placebo [80]. In some countries of the former SU, Md has been used for the treatment of ischemic heart disease, heart failure, infarction, and stroke [79,81,82]. The substance has not been officially approved as a drug in other countries [83,84]. Md has been patented as cardio- and cerebroprotective medication applicable among others for myocardial infarction, angina, and stroke also in combination with the antioxidant Emoxypine (Mexidol), which is not acknowledged as a drug in the United States and European Union [85–90].

The supposed action mechanism of Md is the lowering of carnitine concentration—in muscular, cardiac, neural, and other tissues [91–93]. The oral administration of Md to healthy volunteers at the recommended dose of 500 mg twice daily during 4 weeks caused a significant lowering of plasma carnitine by 18% [94]. Administration of Md 100 mg/kg to rats resulted in a 69% decrease in the L-carnitine concentration in the myocardium [92]. It should be taken into account that carnitine is important for the process of fatty acids transportation to mitochondria coupled with the aerobic synthesis of ATP and accompanied by carnitine etherification and formation of acylcarnitine derivatives. Accordingly, the endogenous carnitine pool consists of carnitine, short- and long-chain acylcarnitines [95–97]. Deficiency of carnitine is associated with fatigue and muscular weakness. There is clinical and experimental evidence that carnitine possesses a cardioprotective effect in patients with cardiomyopathy, angina pectoris, and myocardial infarction [98–103]. In a double-blind study, 2,330 patients with myocardial infarction were randomly assigned to receive placebo or carnitine. The mortality rate was significantly lower in the carnitine group [104]. Carnitine was reported to prevent ischemic injury and losses of high-energy phosphate stores, as well as to improve a heart recovery after reperfusion [100]. Admittedly, the data about benefits from carnitine supplementation are inconsistent [105]. A review concluded that “there appears to be no significant marginal benefit in terms of all-cause mortality, heart failure, unstable angina, or myocardial reinfarction in the setting of acute myocardial infarction for oral carnitine maintenance doses” [106]. Excessive levels of acylcarnitines have been associated with inflammation, ion imbalance, and insulin insensitivity [107–111]; although carnitine supplementation was reported to improve glucose homeostasis in insulin-resistant humans and animal models [106]. Elevated concentrations of acylcarnitines were associated with enhanced cerebro- and cardiovascular risks;

however, confounding factors such as the diet and lifestyle are possible as meat eaters tend to have increased levels of acylcarnitines [112].

Favorable effects of Md have been reported in myocardial infarction, heart failure, stroke, and other conditions [113–118]. In particular, increased tolerance of exercise was observed after the Md intake by patients with heart failure [115]. Similar results have been obtained in animal models [119,120], for example, "statistically significant decrease in the necrotic area" in the brain after the middle cerebral artery occlusion in rats [121]. Considering the diminished availability of ATP as energy carrier due to the lowering of the carnitine level, Md might contribute to the cell damage and decline in the cardiac function in heart failure and infarction. Apparently, the placebo effect has contributed to positive results in some studies.

Propositions about regulatory or ameliorating effects of Md [122] are doubtful as a regulation or amelioration requires a feedback mechanism and cannot be ascribed to a single substance. Furthermore, the following has been proposed: "Carnitine biosynthesis enzyme γ-butyrobetaine hydroxylase and carnitine/organic cation transporter type 2 (OCTN2) are the main known drug targets of Md, and through inhibition of these activities, Md induces adaptive changes in the cellular energy homeostasis. Since carnitine is involved in the metabolism of fatty acids, the decline in its levels stimulates glucose metabolism…" [91]. The benefits from the "glycolysis training," "myocardium training," and "pharmacological preconditioning" [122–124] are hardly applicable for myocardial infarction and heart failure close to decompensation when diminished energy supply can further impair the cardiac function and worsen the outcome. Dambrova and Liepinsh did not comment on these arguments in their authors' reply [108,125]. Supposedly, the World Anti-Doping Agency added Md to the Prohibited List in 2016 because of potential harm to athletes, who may overdose the substance, excessively lowering the carnitine level [83]. There is no compelling evidence that Md is effective in improving athletic performance, nor that its administration is safe for healthy subjects [97,126].

Carnitine and its derivatives are in fact nutritive metabolites available from meat and synthesized by the body, especially in vegetarians. An occasional pharmacological suppression of carnitine concentrations seems to have not much sense for cardiovascular disease prevention; the more so as direct links between acylcarnitine accumulation and human disease have not been established [109]. Potential indications to the lowering of abnormally high carnitine and acylcarnitine levels may become a topic for further research. However, diet modification is not necessarily inferior to the pharmacotherapy as elevated carnitine level can result, e.g., from consumption of meat products. Besides, carnitine is used as a food supplement. In this connection, it is not surprising that carnitine derivatives were reported to be of benefit for people with dementia, nervous disease, and mental disease [127]. Carnitine, carnosine, taurine, various meat-derived peptides, and amino acids are nutrients that would be favorable in conditions of malnutrition and insufficient consumption of meat products, e.g., in some nursing homes, psychiatric facilities, etc. [60]. Moreover, taking into account the biochemical similarity of carnitine and Md, the latter being named a carnitine-mimicking substance or "false carnitine" [84], Md itself can be metabolized as a nutrient.

Finally, peribulbar and subconjunctival injections of Md, patented and applied in the former SU in age-related and vascular eye conditions, diabetic retinopathy and corneal lesions [128–130], should be discouraged. The effect of Md, if any, can be adverse due to the diminished availability of the energy carrier ATP. Undesirable effects might have been masked by a placebo effect known to be associated with invasive procedures. Among known complications of peribulbar injections are hematomas and perforating injuries [73,131]. Peribulbar injections of carcinine and taurine have been commented in the preceding section. In conclusion, at least in some settings, Md acts as a placebo with potential adverse effects, which is named pseudo-placebo [80]. The matter should be clarified in experiments shielded from conflicts of interest.

Conclusion

Examples presented here might appear haphazardly collected, but all of them can be discussed, to some extent, within the scope of scientific misconduct (SM). Since 1998, several cases of SM in medical research in the former SU have been commented, including invasive procedures with questionable indications, trimming of quantitative data, etc. [132–134]. As discussed in this review, a special kind of SM is marketing of placebos and substances with unproven effects under the guise

of evidence-based medications, supported by dubious research. The published examples are only a tip of the iceberg. Considering the ongoing "improvement" of fraudulent skills, scientists, editors, and authorities must jointly combat SM. A response to SM requires national and international bodies to provide leadership and guidelines, while whistleblowers need a safe, confidential place to report SM [135].

Apparently, placebos and other inexpensive substitutes of evidence-based medications are intended by some policymakers for the treatment, among others, of unprivileged elderly people. Such preparations are advertised in Russia and some other countries. A contributing factor might be insufficient theoretic schooling of some physicians, who would prescribe such medications without pondering on mechanisms [136]. The quality of teaching of medicine has been uneven in the former SU. The selling to students of multiple choice tests together with answers has become widespread since the 1980s. Some tests contain ambiguities so that even an expert cannot answer successfully without knowing answers beforehand [137]; more details and references are given in study of Jargin [134]. The author of this review, graduated from Moscow I.M. Sechenov Medical Academy in 1983 and completed postgraduate training of pathology at the same institution, observed that certain courses, e.g., clinical pharmacology or gynecological pathology (for future pathologists) were, in fact, inexistent, in spite of figuring as a part of the curriculum. Students used the Russian-language literature; its quality has been uneven, many books being not up-to-date [136,138]. The access to the foreign professional literature has been limited, medical libraries being in deplorable condition [138]. In fact, we need authorized foreign advisors in different fields of healthcare. There are misgivings, however, that such advisors would be involved in corrupt interactions. However, a non-profit volunteering and clinical attachments of foreign doctors and other specialists are useful as they permit to see internal problems from another viewpoint, which can be helpful in finding solutions [139,140].

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MINI REVIEW

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Pharmacological effects of *Polyalthia cerasoides* (Roxb.) Bedd.: A brief review

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ABSTRACT

Polyalthia cerasoides (Roxb.) Bedd. is also known as *Guatteria cerasoides* or *Uvaria cerasoides* belonging to the family of Annonaceae. *Polyalthia cerasoides* is used in traditional and folklore system of medicine extensively across Asian and African countries for its various pharmacological properties as treatment of toothaches, fever, and combat stress. An exhaustive bibliographic search related to *P. cerasoides* noticed that a large number of bioactive compounds, such as aporphine alkaloids, sequiterpenes, diterpenes, Phenolics, and Isoquinolene compounds enriched the stem bark and roots. The scientific ethnopharmocological studies proved that it possesses a wide range of biological activities such as anti-inflammatory, antioxidant, antidiabetic, antimicrobial, hepatoprotective, anticancerous, analgesic studies, and antistress activities. Isolated bioactive compounds (Cerasoidine, Polyalthidin, laudanidine, codamine, bidebiline E, etc.) exhibits the antimalarial activity against Plasmodium falciparum, antimycobacterial activity against Mycobacterium tuberculosis and anticancer activities have been reported. Its efficacy on diseases proved the future usefulness of different species of *P. cerasoides*. The toxicity studies reveal its non-toxic effect even at larger doses. This review provides the scope of phytochemical, pharmacological, medicinal, and non-medicinal uses of *P. cerasoides*. Further extensive investigation on *P. cerasoides* for its therapeutic potential related to folklore claims and shed the light on its unexplored potentialities.

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Polyalthia cerasoides; bioactive compounds; pharmacological activities; folklore

Introduction

Plants are the richest repositories of diverse phytochemical compounds, which render them to serve as a potential source of bioactive components for the development of therapeutic drugs. A magnitude of change has been observed in usage of medicine, from allopathic toward natural/traditional medicine. Demand for plant-based medicines is increased, owing to its effectiveness, safeness, and restoring the natural ability of the body [1]. The main source for plant-based medicines is the countries like India, Africa, etc., which are rich in biodiversity. India is not only rich in biodiversity but also acquainted with vast knowledge of traditional medicine. Resurgence in the research areas like ethno pharmacology and pharmacology allied to traditional medicine revolutionized the elucidation of novel bioactive components [2]. In search of novel drugs from plants, based on the traditional knowledge, would be considered as a fruitful and promising approach for the development of effective therapeutic drugs than the available ones. *Polyalthia cerasoides* (Roxb.) Bedd. (Annonaceae), is a medium-sized tree (Fig. 1), growing to 10–20 m in height and 20–50 cm in diameter, and is found in mostly in Asian countries [3]. It is locally called as "Gutti dudduga or gutti palla chettu" in the Andhra Pradesh region; this is familiar for its edible fruits [4]. Andhra Pradesh and Tamil Nadu (States of India) tribal people use the fruits and stem bark of the plant used in folklore medicine, while African tribal's use the fruits, roots, and leaves of the plant

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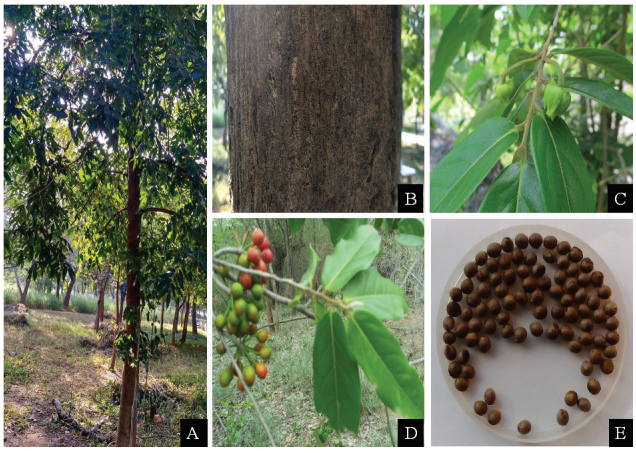


Figure 1. Photographic representation of *Polyalthia cerasoides* (Roxb.) Bedd. (A): Tree, (B): bark, (C): flower, (D): fruits, and (E): seeds.

for treatment of toothaches, fever, as an aphrodisiac, as a deparasitant, and as an anti-inflammatory. Stem bark of *P. cerasoides* reduces the brain stress [5]. The present review summarized the plant profile, phytochemistry, and ethnopharmocological scientific proven activities of *P. cerasoides*. It is also mentioned the need of clinical studies of isolated compounds to develop the novel therapeutic drugs from this natural resource.

Taxonomy and Distribution

Its taxonomy and nomenclature are as follows: Plant name: *Polyalthia cerasoides*; Kingdom: Plantae; Phylum: Tracheophyta; Class: Magnoliopsida ; Order: Magnoliales; Family: Annonaceae; Genus: *Polyalthia*; Species: *cerasoides*. It is mainly distributed in Africa, Burma, China, India, and Thailand [3,6].

Synonyms

Latin: *Polyalthia cerasoides* syn: *Guatteria cerasoides, Uvaria cerasoides*

Common names

Telugu:	Gutti palla chettu/Gutti Dudduga
Hindi:	Kudumi
Tamil:	Nedunarai
Kanada:	Habbe/Sanhesare
Malayalam:	Cherunedunar, Narela

Ethnomedicinal significance

Powder from Stem bark and seeds of *P. cerasoides* used to combat stress by the local medical practitioners of the Tirunelveli district of Tamil Nadu [7]. Stem bark used as a folk medicine by the tribal people of north Odisha to treat diabetes [8]. Root decoction used as traditionally as a tonic and febrifuge by the native people in Thailand [9].

Phytochemical Profile

Bhargavi et al. [6] reported the presence of phytochemical constituents like Alkaloids, Tannins, Terpenoids, Saponins, and Phenols from stem bark extracts. Rawani et al. [10] reported the presence of saponins, steroids, and Terpenoids from the fruit extracts (aqueous).

Bioactive constituents

A large number of bioactive constituents reported from roots and stem (Table 1).

Roots

Aporphine alkaloid-bidebiline E, octadeca-9,11,13-triynoic acid, three sesquiterpenes, α-humulene, Caryophyllene oxide, α-cadinol, four Isoquinoline alkaloids, laudanosine, laudanidine, codamine, reticuline; these nine compounds reported by Kanokmedhakul et al. [11]. Cerasoidine, a Bis-aporphine alkaloid reported by Shono et al. [12].

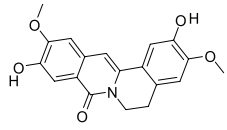
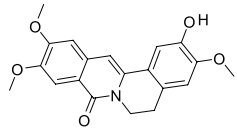
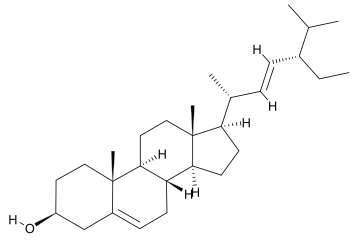
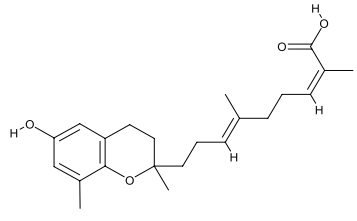
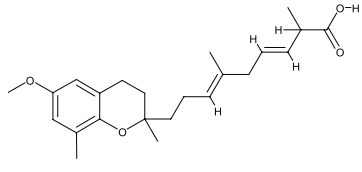
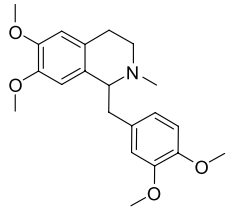
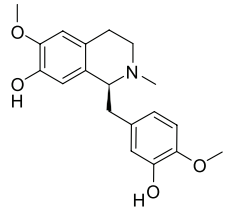
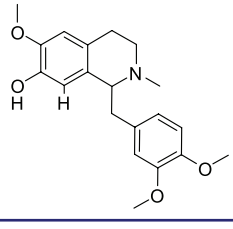
Stem

The liquid chromatography–mass spectrometry studies have been reported the 28 bioactive compounds such as, Humulene, Laudanosine, Reticuline, Maculosine, Retrorsine N-oxide, Delcosine, Ergosine, Thalicarpine, Azetidine 2-carboxylic acid, Isovaleric acid, Methyl amino alanine, Phenethylamine, Methylcytosine, Deoxyquercetin, Acetoxyvalerenic acid, methyl linolenate, Caffeoylmalic acid, Ellagic acid, Hydroxycyanthin, Benzoylmethylecgonine, Eupaformonin, Pelarginidin chloride, Indicaxanthin, Galangin TrimethylEther, Dihydroxy Stearic Acid, Myricetin, Rutacridone Epoxide, Caffeoylshikimic acid; Ethylcetatel extract yielded two oxoprotoberberine alkaloids, cerasoidine, and cerasonine [13,14]; Methanol extract yielded N-4(-hydroxy-B-Phenethyl-4-hydroxycinnamide; Hexane extract yielded stigmasterol; Dichloromethane extract yielded stigmasterol and triterpenes. Polycerasoidin, Polycerasoidol, and Polyalthidin-a benzopyran derivative [15].

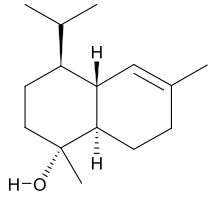
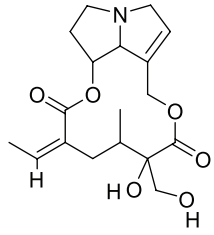
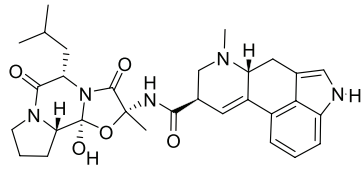
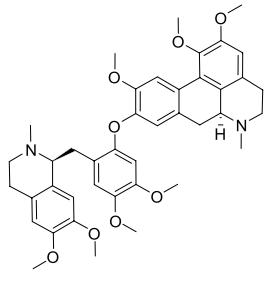
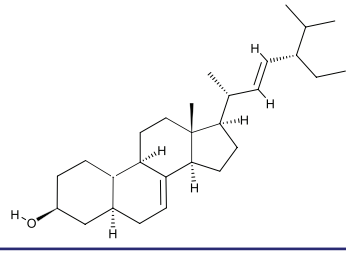
Antibacterial Activity

The extensive use of antibiotics results in the development of antibiotic resistant bacteria, and proved the insufficiency of the available antimicrobial drugs. This rising incidence of antibiotic resistant

Table 1. Phytochemicals/bioactive compounds isolated from the *P. cerasoides* (Roxb.) Bedd.

Phytochemical/compound name	Plant part	Extract medium	Structure	References
Cerasodine	Root, stem	ethylcetatel		[12–14]
Cerasonine	Stem	ehtylcetatel		[14]
Stigmasterol	Stem	Hexane, dichloromethane		[15]
Polycerasoidol	Stem	Benzopyran derivative		[15]
Polyathidin	Stem	Benzopyran derivative		[15]
Laudanosine	Root, stem	hexane, EtOAc, and MeOH		[11]
Reticuline	Root, stem	hexane, EtOAc, and MeOH		[11,13,14]
Codamine	Root	hexane, EtOAc, and MeOH		[11]

continued

Phytochemical/compound name	Plant part	Extract medium	Structure	References
ά- cadinol	Root	EtOAc		[11]
Retrorsine	Stem	Ethanol		[13]
Ergosine	Stem	Ethanol, EtOAc		[13,14]
Thalicarpine	Stem	Ethanol, EtOAc		[13,14]
α-Spinasterol	Seed	Petroleum ether		[33]

bacteria led the researchers to investigate the potent antimicrobial compounds from various medicinal plants. Ravikumar et al. [16] reported, stem bark extracts of *P. cerasoides* exhibited well marked antimicrobial potential against *Pseudomonas*, *Klebsiella*, and *Staphylococcus* (24 clinical strains, 8 of each). All the strains showed more susceptibility to the Ethyl acetate (EA) fraction than compared with Dichloromethane (DCM) fraction. Zone of inhibition for *Klebsiella pneumoniae* (EA 20.12 ± 0.29 mm, DCM 14.30 ± 0.17 mm), *Pseudomonas aeruginosa* (EA 20.00 ± 0.11 mm, 13.52 ± 0.22 mm), and *Staphylococcus aureus* (EA 18.35 ± 0.28 mm, DCM 14.47 ± 0.18 mm). The microbial strains showed that potential susceptibility with ethyl acetate fraction, because of, which consists two berberine alkaloids such as cerasoidin and cerasonine. DCM fraction exhibited less effective response because of benzopyran alkaloids [16].

Rawani et al. [10] reported fruit extracts [Aqueous (Aq) and Chloroform Methanol (CM) (1:1)] of *P. cerasoides* possess antimicrobial potential against four bacterial strains. *Staphylococcus aureus* (Aq 14.03 ± 0.008 mm, CM 30.80 ± 0.57), *Bacillus subtilis* (Aq 15.20 ± 0.15 mm, CM 26.30 ± 0.25), *Escherichia coli* (Aq 18.57 ± 0.32 mm, CM 28.27 ± 0.32 mm), and *Pseudomonas aeruginosa* (Aq 18.37 ± 0.15, CM 30.33 ± 0.24 mm). Chloroform: Methanol (1:1) extracts showed effective antimicrobial response than the aqueous extracts [10].

Minimum Inhibitory Concentration Studies

Treeratnapiboon et al. [9] reported the minimum inhibitory concentration (MIC) studies of Hexane and Dichloromethane extracts from the roots of *P. cerasoides*. Among 27 strains (18 reference strains and 9 clinical isolates), 14 strains are Gram-negative and 11 are Gram-positive bacteria and two are fungal strains. Both the extracts selectively displayed the antigrowth activity against Gram-positive bacteria. Dichloromethane extract exhibited the highest activity against *Corynebacterium diphtheriae* with MIC of 32 μg/ml and the least with *Bacillus cereus* (128 μg/ml) and *Micrococcus luteus* (256 μg/ml) [9].

Acute Toxicity Studies

Goudarshivananavar et al. [17] analyzed the toxic effect of *P. cerasoides* extracts at various concentrations 50, 100, 200, 500, 1,000, 2,000 mg/kg b.w. and reported no toxicity of plant extracts even at 2,000 mg/kg b.w.

Pharmacological Activities of *P. cerasoides*

Polyalthia cerasoides have the several pharmacological activities, proved by scientific observations of experimental works. The bark, leaves, and fruits are extensively used in traditional medicine due to the presence of several phytoconstituents like alkaloids, terpenoids, saponins, and flavonoids. Scientific evaluations of isolated bio-compounds have ethnomedicinal and novel pharmacological effects. Table 2 represents the pharmacological findings obtained from various parts of the plant in different solvent extractions.

Antidiabetic Activity

Diabetes is a metabolic disorder that occurs due to insulin imbalance production. Some of the physiological or developmental factor effects on pancreatic beta cells cause hormonal imbalance probably diabetes [18]. Current lifestyle, such as mistimed sleeping, shift work, or eating at abnormal night-time hours, have been related to type 2 diabetes, obesity, and metabolic syndrome [19]. The number of people who have diabetes has raised steeply more than 371 million persons globally, and is projected to affect 522 million people by the year 2030 [20]. In the developing countries, phytotherapy play a prominent role in the management of the disease for some decades. Identification of plant materials that can manage diabetes and its complications would save millions of people [21]. A study was conducted to test the antidiabetic activity by *P. cerasoides* stem bark. After treating streptozotocin (STZ) diabetic rats for 21 days (chronic study) with a single dose of pcEE (400 mg/kg bw) was shown an effective antidiabetic role by significantly lowering the fasting blood glucose (FBG) levels in diabetic rats. The decrease in blood glucose levels was from 349 ± 7.7 mg/dl (Diabetic rat) to 168 ± 6.4 mg/dl (control) from 0 to 21 days' time period. The decreasing glucose levels were nearly similar to the effect of standard drug glibenclamide (341.8 ± 7.8 mg/dl to 159.3 ± 6.3 mg/dl). However, acute exposure of different plant extraction in n-hexane, ethyl acetate, ethanol, and aqueous extracts of *P. cerasoides* stem bark to 12 hours fasted normal and STZ induced diabetic rats at a dose of 200, 400, and 600 mg/kg bw for acute studies. Among four extracts, PcEE and PcEAE (400 mg/kg bw) showed effects on blood glucose levels. However, PcEAE showed the least effect on the reduction of blood glucose compared with PcEE. The remaining two extracts did not show any positive effect in FBG levels. Nearly, 400 mg/kg bw of pcEE treated showed the potential increasing the body weights (179.1 ± 4.9 to 185.5 ± 8.3) but diabetic rats showed the decreased body weights when compared with control rats. Liver and kidney morphological changes were prevented by pcEE (400 mg/kg bw) administration compared with diabetic rats. It clearly reveals that *P. cerasoides* has the antidiabetic effect [6]. Bhargavi et al. [22] reported the hypolipidemic effect of the stem bark ethanolic extract at 400 mg/kg bw. Changes the lipid profile (lowered the total cholesterol, triglycerides, low-density lipids, and very low density lipids) and serum biochemical marker enzymes like, aspartate transaminase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in STZ induced rat models [22].

Increase the levels of serum lipid profile levels, such as cholesterol, triglycerides, low density lipoprotein (LDL), very low density lipoprotein levels, and decreasing levels of high density lipoprotein (HDL) levels in the diabetic rats may be due to insulin activates lipoprotein lipase and hydrolysis of triglycerides [23]. Insulin increases uptake of fatty acids into adipose tissue and increases triglyceride synthesis. HDL is an anti-atherogenic lipoprotein [24]. The level of HDL-cholesterol slightly increased after the administration of ethanolic extract of *P. cerasoides* stem bark at 400 mg/kg bw [22]. This might be due to increase in the activity of lecithin

Table 2. Pharmacological activities of *P. cerasoides* (Roxb.) Bedd.

Activity	Plant part	Extraction medium	Against toxic factor	Animal modal/mode of study	Dosage	Administration route	Result	Reference
Anti stress activity	Stem bark	Ethanol	Cold immobilization stress	Albino rat/*in vivo*	100 mg/kg bw	Gastric Intubation.	Increase the levels of monoamine oxidase (MAO), decreasing the elevated levels of 5-HT and 5-HIAA induced by the stress.	[7]
Anticancerous/ Antipropliferative	Seeds	Petroleumether extract	Methylmethane sulfonate	Swiss albino mice/ CACO-2 cell lines	5–10 mg/kg bw	I.P (Intraperitoneal)	The compounds Clerodane diterpenoid, Spinasterol, and α-Spinasterol showed antiproliferative action on CACO-2 cell line and also showed anticancerous activity against the MMS-induced mutagencity in albino mice.	[33]
Hepatoprotective	Stem bark	Ethyl acetate	CCl_4	Albino rat/*in vivo*	250 and 500 mg/ kg bw	P.O (orally)	SOD and CAT levels increased, elevated levels of LPO reduced to normal	[17]
	Stem bark	Ethanolic extract	CCl_4	Albino rat/*in vivo*	100 mg/kg bw	Gastric intubation.	Elevated levels of serum and tissue SGPT, SGOT, and Alkaline phosphates significantly reduced, total proteins levels increased, and lipidperoxidation levels decreased	[29]
Antioxidant	Stem bark	Alcoholic		Swiss albino mice and *in-vitro* studies	100 mg/kg body weight	orally	DPPH, hydroxyl radicals, Superoxide anion scavenging, reducing power assays scavenging by P.c bark extract	[32]
Anti-inflamatory	Stem bark	Ethyl acetate/ petroleum ether	Carrageenan	Albino rat	100 and 200 mg/ kg bw	p.o	Ehtylacetate fraction inhibits the paw edema by 57.38% and 68.5% when compared with petroleum ether fraction (26.22% and 36.06%). Ethyl acetate fraction effective inhibition and similar to the effect of standard drug Diclofenac (75.4%)	[17]
Analgesic activity	Stem bark	Ethyl acetate/ petroleum ether	Acetic acid	rat	100 & 200mg/ kg bw	P.o	Ehtylacetate fraction reduces the pain by 61.73% and 63.68% compared with petroleum ether (28.2% and 48.8%), ethyl acetate fraction significantly reduced the pain as standard drug diclofenac (63.75%)	[17]
Antidiabetic	Stem bark	Methanol	STZ	Rat	200 and 400 mg/ kg bw	Oral	FBG levels significantly lowered in PcEE group compared with STZ-treated group.	[6]
	Stem bark	Ethanol	STZ	Rat	400 mg/kg bw	oral	Serum marker enzymes ALT, AST, and ALP levels lowered, and lipid profile levels [total cholesterol, TG, LDL, and (acetylated low density lipoprotein (ALDL))] retained to normal and HDL levels not changed.	[22]

cholesterol acyl transferase, which may contribute to the regulation of blood lipids. AST, ALT, and ALP are considered as a part of liver toxicity markers. In streptozotocin-induced diabetic animals, change in the serum enzymes is directly related to changes in the metabolic functions of AST, ALT, and ALP. It has been reported that the increased aminotransferase activities under insulin deficiency [25] were responsible for the increased gluconeogenesis and ketogenesis during diabetic. The mechanism, by which the serum aspartate and alanine aminotransferases are raised in diabetic untreated, may involve increased liberation of these enzymes from tissues (mainly liver), owing to oxidative stress or the formation of advanced glycosylation end product [26]. The increase in the activities of these enzymes in serum of diabetic control might be induced due to liver dysfunction.

Hepatoprotective Activity

Hepatic diseases are one of the most serious and common disease to the mankind. Pathogenesis of the hepatic diseases is due to the oxidative stress and the inflammation [27]. Despite, tremendous advances in modern medicine, the management of liver disease is still a major challenge [28]. A promising hepatoprotective activity with stem bark extracts of *P. cerasoides* was evidenced against Carbon tetrachloride (CCl_4)-induced hepatotoxicity in albino rats. Alcoholic extract of 100 mg/kg bw reduces the lipid peroxidation and serum phosphate levels in the liver. Liver marker enzymes glutamic oxaloacetic transminase [(serum glutamic oxaloacetic transaminase (SGOT)), AST], glutamic pyruvic transaminase [(serum glutamic pyruvic transaminase (SGPT)), ALT], and alkaline phosphatase levels were significantly increased in blood serum as well as liver tissue in the CCl_4-treated rats when compared with the control. The elevated levels of key marker enzymes were reduced to normal levels in the alcoholic extract *P. cerasoides* (100 mg/kg bw for 7-day treatment). Decreased total protein levels and increased (lipid peroxidation (LPO)) levels were neutralized in the plant extract administered group of rats, indicating that the plant extract may scavenges the reactive oxygen species produced by CCL_4 metabolism in which could be acted as hepatoprotective drug agent [29]. Goudarshivananavar et al. [17] reported the ethyl acetate fraction at dose 250 and 500 mg/kg bw significantly improved the levels of liver antioxidant enzymes such as Catalase (368.2 ± 1.54 and 398.4 ± 5.23), Superoxide dismutase (SOD) (15.11 ± 1.58 and 19.54 ± 3.22), and peroxides (118.78 ± 5.12 and 131.32 ± 4.30), when compared with CCl_4-induced hepatic rats (121.54 ± 1.53, 8.48 ± 0.12, and 48.43 ± 2.70 units/mg). Increased depleted antioxidant enzyme levels in the liver tissue may prove that the plant extract protecting the structural integrity of hepatic cells or reconstruction of necrotic hepatic cells. Effectiveness of the plant extract was similar to the standard drug Silymarin [17]. These findings suggest the presence of potential bioactive components to normalize the antioxidant enzymes that are involved in combating reactive oxygen species (ROS), and thus protecting the structural integrity of hepatocyte cells.

Antioxidant Activity

Oxidative stress causes the generation of free hydroxyl radicals and ROS have been implicated in degenerative/pathological process. Free radicals aroused during the stress have a broad range of effects in biological systems [30]. Plant-based medicines serve as an excellent antioxidant because of the presence of various phenolic contents. Natural Plant-based antioxidants protect from the damaging effect of oxidative stress by quenching the ROS and OH^- free radicals, and therefore, useful in the treatment of cancer, cardiovascular, and anti-inflammatory diseases [31]. Ravikumar et al. [32] analyzed the antioxidative potential of alcoholic bark extracts of *P. cerasoides* by using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The hydroxyl radical, Superoxide anion scavenging, and reducing power assays were reported the dose-dependent inhibition of DPPH scavenging activity and indicates 50% inhibition rate. This concentration was designated to 0.589 µg/ml of tannic acid/mg of plant extract equivalency. The significant antioxidant potential of *P. cerasoides* extracts might be attributed to the presence of polyphenols (Hydroxycinnamic acid and Ellagic acid). This study shed the light on the potential antioxidant properties of *P. cerasoides* and supports its use to develop potent antioxidant drugs [32]. Ethylacetate fraction exhibited DPPH radical (1–100 µg/ml) scavenging activity with inhibitory concentration (IC) 50 is about 42.43 µg/ml, whereas the IC value of standard ascorbic acid (1–5 µg/ml) was 3.19 µg/ml [17].

Anti-Stress Activity

Padma et al. [7] noticed the anti-stress capability of *P. cerasoides* stem bark alcoholic extracts. Albino

rats were used as models for the study. Cold immobilization stress induced in rats after treating with plant extracts. Stress causes the rise in the level of enzymes like Nor-epinepephrine, dopamine, 5-hydroxytryptamine (5-HT), 5-hydroxy Indole acetic acid (HIAA) in control group rats. Plant extract treated group, plant extract at dose 100 mg/kg bw for a period of 16 days normalized the levels of all the enzymes. Pretreatment with plant extracts resulted in increasing the level of Monoamine oxidase and reduced the levels of other enzymes induced by stress. The elevated level of monoamine oxidase by the plant extracts indicates its adaptogenic potential. This study authenticates the use of *P. cerasoides* stem bark by the folklore (Tirunelveli district, Tamil Nadu) as a tonic to combat the condition of stress [7].

Anticancerous/Antiproliferative Studies

A number of plant-based medicines currently used as effective anticancerous agents like vinblastine, vincristidine, paclitaxol, bleomycin, cisplatin, prednisome, and procarbasome. Ravikumar et al. [33] reported antiproliferative effect of isolated compounds spinasterol and clerodane diterpenoid on CACO-2 cell lines. Clerodane diterpenoids induce apoptosis (cell death) effective at lower concentrations. Spinastrerol and α-spinasterol showed an antiproliferative effect in a dose-dependent manner. A significant activity was observed at 30, 60, and 80 nm compared with the reference standard drug paclitaxol. Clerodane diterpenoid, Spinastrerol, and α- spinasterol exhibited Antiproliferative action at various concentrations with an IC_{50} value of 28.6 + 4.54 nM/ml, 57.7 + 6.81 nM/ml, 60.0 + 7.10 nM/ml [33]. Banjerdpongchai et al. [34] recently proved the anticancer property of *P. cerasoides*. The purified compound the 6, 8-dihydroxy-7-methoxy-1-methyl-azafluorenone (DMMA) isolated from roots of the plant. The inhibitory concentrations at 20% and 50% (IC_{20} and IC_{50}) of DMMA toward Human Cancer Cells HL-60 (18.7 and 46.7 μM), U937 (11.7 and 29.2 μM), MOLT-4 (14.0 and 35.0 μM), HepG2 (7.4 and 20.1 μM), MDA-MB231 (16.7 and 55.6 μM), and PBMCs (12.4 and 31.1 μM). DMMA inhibited the five human cancer cell proliferations in a dose-dependent manner from (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)) assay, HepG2 cells were the most sensitive to and MDA-MB231 cells were the most resistant against DMMA-induced Cytotoxicity as showed by the IC_{50} levels [34]. Clerodane diterpenoids was induced the apoptosis by interfering with topoisomerase-II inhibition, whereas phytosterols induce apoptosis by BCL2 Associated X (Bax) apoptosis regulator protein activation [35]. This study authenticates the use of diterpenoids and phytosterols from the *P. cerasoides* seeds to develop effective anticancerous drugs in near future.

Anti-Inflammatory Activity

Polyalthia cerasoides stem bark extracts possess significant anti-inflammatory activity. Carrageenan-induced paw edema in Swiss albino rats used to explore the anti-inflammatory effect of the plant extract. Ethyl acetate fraction at dose 100 and 200 mg/kg bw inhibited the paw edema by 57.38% and 68.85%, and the petroleum ether fraction was 26.22% and 36.06%. Ethyl acetate fraction exhibited effective inhibition and it is similar to the effect of standard drug diclofenac (75.4%) [17].

Analgesic Activity

Goudarshivananavar et al. [17] analyzed the analgesic property of *P. cerasoides* petroleum ether and ethyl acetate extracts against acetic acid induced writhing model and reported the effectiveness of ethyl acetate extract at dose 100 and 200 mg/kg bw reduced the pain by 61.73% and 63.68%, whereas petroleum ether reduced the pain by 28.2% and 48.8%. Ethyl acetate fraction showed significant reduction of pain, similar to standard drug diclofenac (63.75%) [17].

Antimalarial Activity/Mycobacterium Activity

Malaria is the major parasitic disease, mostly found in tropical countries and other countries. Present days, it leads to major global public health problem spread of drug resistance and limited number of effective drugs [36]. This necessitates searching the safe and effective antimalarial drugs alternative to the existing ones. Traditional medicinal knowledge yields the anti-malarial drugs like quinine and artemesin and their efficiency to control malaria, stimulated many researchers to find the similar potential anti-malarial drug from the plant sources [37]. Kanokmedhakul et al. [11] reported the antimalarial activity against the *P. falciparum* (K1, multidrug-resistant strain). The isolated compounds bidebiline E(1), octadeca-9,11,13-triynoic acid (2), caryophyllene oxide (4), codamine (7), and

laudanidine (8) from *P. cerasoides* root extraction (various solvents) exhibits the antimalarial activity. Compounds 1, 2, 4, 7, and 8 showed an inhibitory concentration of 50% reduction in parasite growth of *P. falciparum* were 4.2, 5.0, 2.8, 4.2, and 7.0 μg/ml; among these, compounds 8 and 2 showed efficient antimalarial activity and compounds 1 and 7 exhibit equal inhibitory concentration (IC_{50}) but compound 4 showed the moderate IC_{50}. Compound 3 (alpha-humulene) did not show any antimalarial activity [11].

Compounds 1, 2, and 3 possess the Antimycobacterial activity against *M. tuberculosis* H37Ra using the microplate Alamar Blue assay with reference standard drugs isoniazid and kanamycin sulfate. MIC of compound 1 showed 6.25 μg/ml, compound 2 was 6.25 μg/ml, and compound 3 represents with same MIC (6.25 μg/ml) as 1, 2 compounds, whereas compounds 4, 7, and 8 did not show any inhibitory action against the *M. tuberculosis*, they showed inactive action [11].

Conclusion

Polyalthia cerasoides stem bark and fruits are used by the folklore as a traditional medicine to combat the condition of the stress. Phytochemical screening revealed the presence of tannins, phenols, alkaloids, triterpinoids, and saponins. However, the seeds possess the sterols. Pharmacological studies revealed the significant effect of plant extracts similar to that of standard drugs. The largest number of compounds is isolated from root and stem barks but the pharmacological studies on isolated compounds are limited. However, the isolated bioactive compound like 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone showed the antiproliferative effect on human cancer cells. Codamine, laudanidine, bidebiline E, and caryophyllene oxide showed very efficient antimalarial activity against the *P. falciparum*. Even though it is used as a traditional medicine for combating stress, for a long time, only the effect of crude extract was reported. Present review highlighted the pharmacological activities reported from previous studies, and stressed the need of pharmacological and clinical studies to evaluate the effectiveness of bioactive compounds from *P. cerasoides*. This review provides the scope for further investigation of unexplored potentialities (anti-stress compounds) and the possibility to develop the novel and the most effective anti-stress medicine.

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ORIGINAL RESEARCH

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Stability indicating high-performance thin-layer chromatography method for estimation of ascorbic acid in *Hibiscus sabdariffa* L. aqueous extract

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ABSTRACT

Background: High-performance thin-layer chromatography (HPTLC) has become a routine analysis technique due to its advantages of low operating cost, high sample throughput, and need for minimum sample clean up.

Aim: To develop and validate an accurate, specific, repeatable, and stability-indicating HPTLC method for the determination of ascorbic acid in presence of their degradation products for assessment of stability of the ascorbic acid in *Hibiscus sabdariffa* L. preparations.

Methods: Chromatographic sample separations employed thin layer chromatography plates (20 cm × 10 cm) pre-coated with silica gel 60 F254 as a stationary phase. Solvent system consisted of acetone:toluene:water:glacial acetic acid (26:4:4:1 v/v) while scanning wavelength of 269 nm was used. Ascorbic acid was subjected to different stress conditions, i.e., oxidation, dry heat treatment, photodegradation, and hydrolysis under different pH. The method was validated according to International Council for Harmonisation guidelines.

Results: Peaks of products formed were well resolved with different R_f values. The linear regression data for the calibration plots showed good linear relationship with $r^2 = 0.9912$ in the concentration range of 2–4 µg/ml. The mean value slope and intercept were −531.72 and 8,040.22, respectively.

Conclusion: A rapid, simple, accurate, and specific HPTLC method for determination of ascorbic acid in *H. sabdariffa* L. preparations was developed and validated. In addition, this method separated all the degradants from stress conditions showing that it is a stability indicating method. The developed method could now be used in quality control of *H. sabdariffa* herb products.

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Ascorbic acid; chromatography; *Hibiscus sabdariffa*; quality control

Introduction

Ascorbic acid, popularly called vitamin C (Molecular weight: 176.124 g/mol; Molecular formula: $C_6H_8O_6$), has many biological activities in the human body. It is best known as potent enhancer of iron absorption from gastrointestinal tract when taken naturally in both fruit and vegetables [1] and when added as the free compound [2]. In human diets, more than 90% of the vitamin C is supplied by fruits and vegetables, including potatoes [3]. It is also among the important constituent of *Hibiscus sabdariffa* L. calyces [4]. In aqueous solutions, vitamin C easily gets oxidized. The oxidation process is greatly favored by the presence of oxygen, heavy metal ions, especially Cu^{2+}, Ag^{+}, and Fe^{3+} and by alkaline pH, as well as high temperature [5].

The abundant presence of vitamin C along with other chemical and mineral constituents in *H. sabdariffa* L. calyces has contributed to its widespread use for various medical conditions [6–8]. Concerned by the growing popularity of

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using *H. sabdariffa* L. and the known unstable nature of vitamin C in aqueous solution, as well as the economic status of *H. sabdariffa* L. users, the need for a simple and affordable method that could clearly indicate stability of ascorbic acid in various *H. sabdariffa* L. products was felt.

Many analytical techniques have been suggested for the detection of ascorbic acid in varieties of samples, including sensors and biosensors [9,10]. Hyphenated instruments consisting of flow injection analysis [11–13] and high-performance liquid chromatography (HPLC) [14,15]. However, these methods are time-consuming, costly, and need special training to operate them.

High-performance thin layer chromatography (HPTLC) has become a routine analysis technique due to advantages of low operating cost, high sample throughput, and need for minimum sample clean up. The major advantage of HPTLC is that several samples could be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis.

The aim of the present work was to develop an accurate, specific, repeatable, and stability-indicating HPTLC method for the determination of ascorbic acid in presence of their degradation products for assessment of stability of the ascorbic acid in *H. sabdariffa* products. The proposed method was validated as per the International Council for Harmonisation of technical requirements for pharmaceuticals for human use (ICH) guidelines [16].

Materials and Methods

Chemicals and reagents

Standard ascorbic acid was obtained as a gift from Zenufa laboratories Ltd (Tanzania). Methanol, ethyl acetate, toluene, acetone, and acetic acid (glacial) solvents were of analytical grade obtained from Carlo Erba Reagents (Italy). In this experiment, distilled water used was in-house prepared.

Plant materials

Calyces of *H. sabdariffa* L. (Malvaceae) were collected from local farms in Dodoma in March 2014. Botanical identification was done by a plant taxonomist and a voucher specimen number 4992 was deposited at the herbarium in the botany department at University of Dar es Salaam, Tanzania. The plant name has been checked with www.theplantlist.org to verify its botanical data during identification. The calyces were then sun-dried and inspected for any extraneous matter prior to extraction process.

Preparation of aqueous extract (juice)

Aqueous extract was prepared based on the optimized extraction parameters for both iron and ascorbic acid as detailed elsewhere [17].

Instrumentation

The experiment used HPTLC Camag Linomat V applicator (Muttenz, Switzerland), a Camag trough chamber, Camag TLC scanner III, Wincats (version 1.4.3) software as data integrator, and a Hamilton syringe (Switzerland) of 100 µl capacity. The TLC plates used for chromatographic sample separations (20 cm × 10 cm) were pre-coated with silica gel 60 F254 (Merck, Darmstadt, Germany).

Stock standard solution (1 mg/ml)

Twenty-five milligrams of ascorbic acid was weighed into 25 ml volumetric flask. The volume was made up to the mark with 50% v/v methanol solution. The flask was closed and shaken well to dissolve all ascorbic acid. It was then labeled ascorbic acid stock standard solution.

Working standard solution (0.5 mg/ml)

Five milliliters of the stock solution was pipetted into 10 ml volumetric flask. The volume was made up to the mark with 50% v/v methanol solution. The flask was closed and then labeled ascorbic acid working standard solution.

Preparation of sample

Five milliliters of standardized *H. sabdariffa* juice was placed in 10 ml volumetric flask and made up the volume with 50% v/v methanol. Sonicated for 5 minutes and filtered through Whatmann filter paper No. 1.

Preparation of mobile phase and developing tank

Mobile phase used was acetone:toluene:water:glacial acetic acid (26:4:4:1). About 26 ml of acetone, 4 ml of toluene, 4 ml of water, and 1 ml of glacial acetic acid were measured using a measuring cylinder and poured into a 50 ml volumetric flask. The flask was then closed and shaken well to ensure thorough mixing.

Developing tank saturation was done by cutting a filter paper and fitting into one side of the developing tank. Then, the mobile phase was poured into the developing tank by wetting the whole filter

paper and the lid was closed immediately. The tank was then left for 25 minutes to saturate before developing.

Challenging tests

The stress degradation studies including hydrolytic in both acidic and alkali medium, photolytic, oxidative, and dry heat induced studies were performed for active ascorbic acid and standardized *H. sabdariffa* juice as per ICH guidelines [16]. A stock solution containing 5 mg ascorbic acid in 10 ml methanol was prepared for forced degradation to indicate the stability indicating property and specificity of the proposed method. In all degradation studies, 2,500 ng per spot was applied in five replicates.

Method validation

Precision

Repeatability and intermediate precision were performed by independently weighing six replicate samples powder equivalent to 0.5 mg/ml ascorbic acid concentration that corresponded to 100%. Intermediate precision was done using two analysts on different days. Weighing scale was calibrated before using, sample preparation and dilutions to the corresponding concentrations were monitored. The mean, standard deviation, and percentage relative standard deviation (%rsd) of peak responses were evaluated. The limit of %rsd of <2% for repeatability and <2% for intermediate precision was considered as acceptable limits.

Specificity

Specificity was evaluated by spiking standard ascorbic acid into *H. sabdariffa* juice to examine possible interference with the analyte peaks during chromatographic run. The method was to be accepted if no interference was observed between analytes and solvents densitograms. Limit of detection (LOD) and limit of quantitation (LOQ) were also calculated.

Linearity and range

Preparations were made by weighing 50 mg of ascorbic acid into a 50 ml volumetric flask to obtain a stock solution. From the stock solution, serial dilutions were prepared to obtain five levels that had 80%, 90%, 100%, 110%, and 120%. This was achieved by accurately measuring separately 4, 4.5, 5, 5.5, and 6 ml of the stock solution into a 10 ml volumetric flask. Concentrations were spotted as 2,000–3,000 ng/spot. Calibration curves plot of peak responses versus concentration was made. The procedure was repeated for three consecutive days. Analysis of variance test was performed to test fitness of the calibration model.

Accuracy

Accuracy was evaluated using standard addition recovery method by spiking ascorbic acid into *H. sabdariffa* juice. Three solutions of the controls were prepared at each level of the concentration at 80%, 100%, and 120% by independently weighing each analyte in triplicate.

Robustness

Robustness of the method was done by deliberate varying parameters such as saturation time, mobile phase composition, and developing chambers. Except for reagent and developing tank, other parameters were varied in a range of ±5% and the test was observed for change in R_f.

Results and Discussion

Stability-indicating HPTLC studies of the samples obtained during stress testing of ascorbic acid under different conditions using acetone:toluene:water:glacial acetic acid (26:4:4:1 v/v) as the mobile phase are presented below. Figure 1 shows the chromatogram of pure ascorbic acid (R_f value 0.49). The mobile phase so developed achieved good resolution of ascorbic acid. Identification of ascorbic acid from *H. sabdariffa* juice was done by comparing chromatogram of standard with that of juice using the established optimized chromatographic conditions (Table 1).

Acid-induced degradation

Acid degradation studies showed the presence of one extra peak other than that of ascorbic acid, which shows its peak at R_f value 0.49 (Fig. 2). The extra peak could represent the formation of degraded product.

Base-induced degradation

The ascorbic acid was also found to undergo alkaline degradation. The reaction in 0.1 mol sodium hydroxide showed presence of one peak at different R_f value 0.54 than that of ascorbic acid (Fig. 3). The extra peak confirm the formation of degradation product.

Hydrogen peroxide-induced degradation

Ascorbic acid was highly degraded in 3% hydrogen peroxide at room temperature. Hydrogen peroxide-induced degradation showed two extra peaks (Fig. 4).

Linearity

Calibration plot shown in Figure 5 indicates that the response was linear function of concentration in the range of 2–4 µg for ascorbic acid. The correlation coefficient, intercept, and the slope for ascorbic acid were 0.99119, 770.353, and −5331.72, respectively.

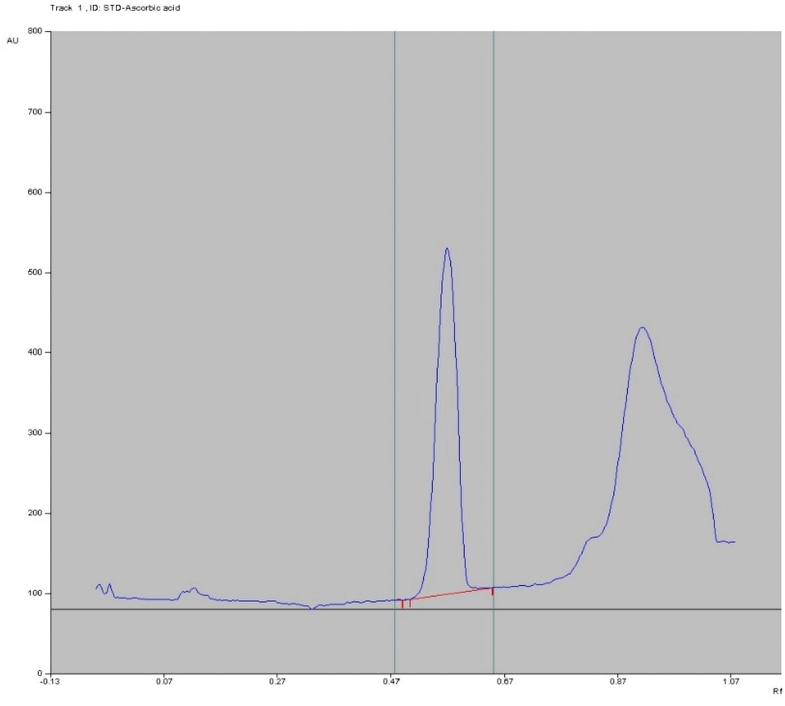

Figure 1. The HPTCL chromatogram of pure ascorbic acid.

Table 1. Optimized chromatographic conditions.

Parameter	Condition
Mobile phase	Acetone:toluene:water:glacial acetic acid (26:4:4:1) v/v
TLC plate	TLC (20 × 10) cm, silica gel 60 F 254
Diluent	Methanol 50%
Saturation time	25 minutes
Developing time	7–10 minutes
Applicator	Linomat 5, a semi-auto sampler applicator
Solvent front position	7 cm
Detector	Densitometry
Detection wavelength	269 nm
Temperature	24°C–30°C
Relative humidity	34%–55%
Application volume	5 µl
Developing tank internal parameters	(20 × 10) cm
Band length	6 mm
Slit dimension	6.00 × 0.45 mm
Syringe volume	100 µl

Since the calibration curve is linear, the slope represents a measure of sensitivity; how much the signal changes for a change in concentration. A steeper line with a larger slope obtained indicates a more sensitive measurement.

Precision

The peak area was measured in triplicates for inter-day and intra-day. The results showed that %RSD was <2%, indicating the acceptable precision of the method (Table 2).

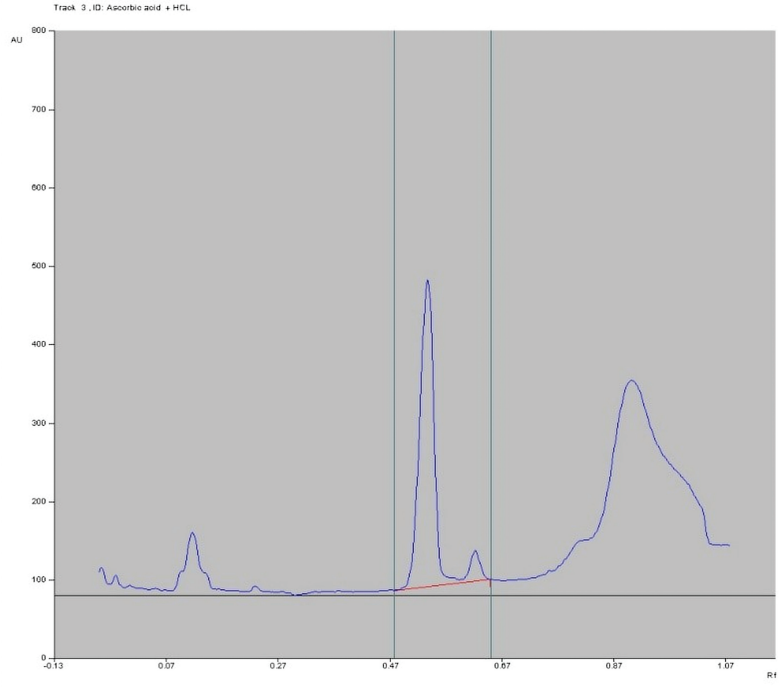

Figure 2. The HPTCL chromatogram of acid degraded ascorbic acid (0.1 M HCL).

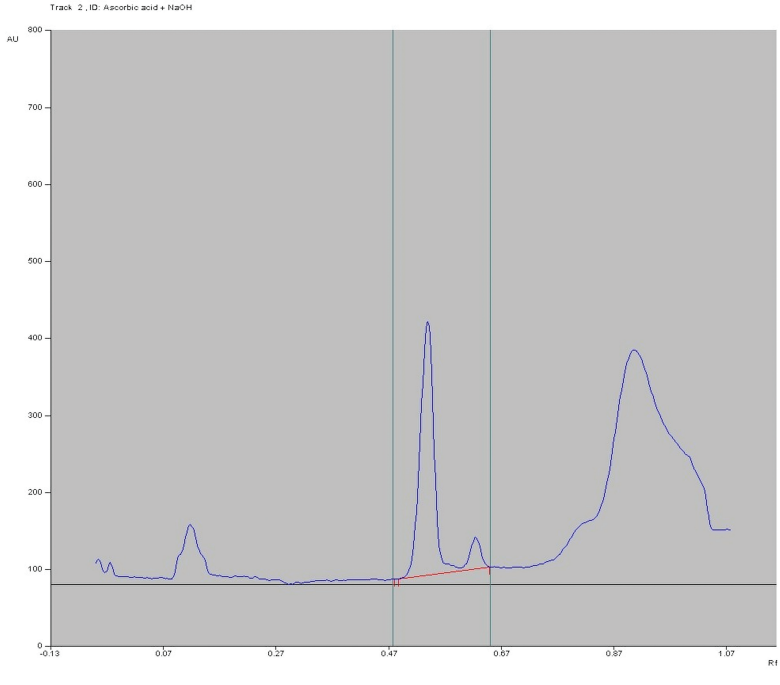

Figure 3. The HPTLC chromatogram of base degraded ascorbic acid (0.1 N NaOH).

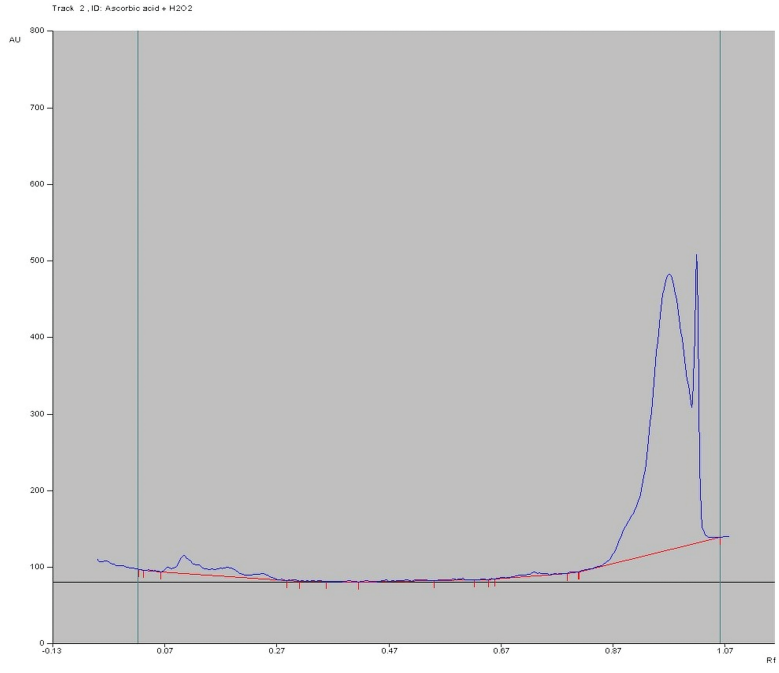

Figure 4. The HPTLC chromatogram of 3% hydrogen peroxide degraded ascorbic acid.

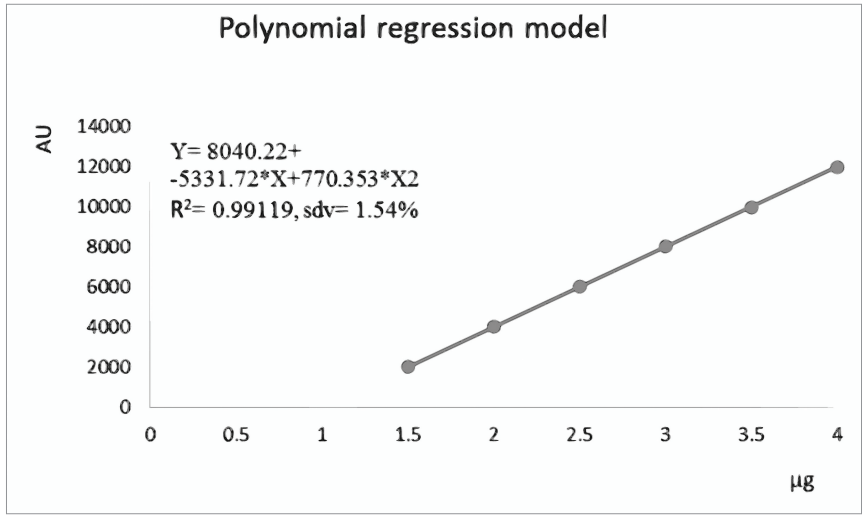

Figure 5. Calibration curve.

The results of LOD and LOQ showed that the method so developed is sensitive (Table 2). It was observed that the method was able to separate all degradants and was not interfered with other constituents in the *H. sabdariffa* juice. Hence, the method was specific. However, it is worth noting that, in the presence of 3% hydrogen peroxide, ascorbic acid undergoes complete degradation.

Robustness

When changes were made on saturation time and development tank, there was a slight change of R_f value. The small change observed could indicate the robustness of the method developed.

Conclusion

The developed HPTLC technique is precise, specific, accurate, and stability-indicating. The analysis proved that the method is suitable for the analysis of ascorbic acid as pure drug and in *H. sabdariffa* L. aqueous extract (juice) without any interference from the excipients. Because the method separates the drug from its degradation products, it can be

Table 2. Summary of analytical results for method validation.

Method characteristics	Ascorbic acid	Comment
Linearity performance parameter		
Linearity range	2–4 µg	Good liner correlation
Correlation coefficient	0.99119	
LOD	0.89951	
LOQ	0.28420	
Accuracy (% Recovery)		
80%	97.27% ± 0.29103%	
100%	97.84% ± 0. 30167%	
120%	97.96% ± 0.28510%	
Precision (%RSD)		
Intraday-Analyst 1 (Day 1)	1.51%	Less than 2% OK
Intraday-Analyst 2 (Day 2)	1.73%	Less than 2% OK
Interday-Inter-analysts	1.61%	Less than 2% OK
Robustness		
Normal R_f	0.49	
Over 24 hours saturation time R_f	0.53	Increased R_f
Challenge		
Normal	R_f – 0.49	Separated
0.1 N HCL	R_f – 0.54	Separated
0.1 NaOH	R_f – 0.54	Separated
3% H_2O_2	Two peaks seen	Separated
=Thermal (70°C)	R_f – 0.55	Separated

used as a stability-indicating method. Therefore, it is proposed for the analysis of ascorbic acid from *H. sabdariffa* L. in presence of degraded products of samples obtained during processing.

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Conflict of Interests

The authors declare that there is no conflict of interest in the study.

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ORIGINAL RESEARCH

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Kolaviron mitigates proteinuria and potentiates loop diuresis in Wistar rats: Relevance to normal renal function

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ABSTRACT

Aim: This study investigated the effects of oral kolaviron administration on proteinuria in Wistar rats.

Methods: The study used 20 Wistar rats that were divided into four groups of five rats each. Group 1 orally received 2 ml/kg of propylene glycol, for 30 consecutive days before they were sacrificed. Groups 2, 3, and 4 received graded doses of kolaviron at 100, 200, and 400 mg/kg (p.o.), respectively, for 30 consecutive days before they were also sacrificed.

Results: Graded doses of kolaviron (100, 200, and 400 mg/kg p.o.) significantly lowered urine total protein and urine total protein–creatinine ratio with >80% reduction when compared with the control. However, creatinine clearance was significantly increased, while the fractional excretions of Na^+ and urea were significantly lowered. While plasma electrolytes were normal, the urinary excretion of Na^+, K^+, and Cl^- were significantly increased in the kolaviron-treated groups. Urine output of the treated rats was significantly higher than that of the control without increase in both plasma and urine glucose level. Renal function biomarkers (creatinine and urea) of the rats indicated a kolaviron-enhanced improvement of renal function with an associated increase in their urine excretion. Micrographic evidence showed no apparent distortion of the kidney histoarchitecture following kolaviron administration to the rats.

Conclusion: This study concluded that kolaviron mitigated proteinuria and potentiated loop diuresis in Wistar rats, a pharmacological benefit that can be utilized for the management or treatment of nephropathic conditions with associated proteinuria.

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Kolaviron; loop diuresis; proteinuria; Wistar rats

Introduction

A loop diuretic refers to any substance that can increase urinary excretion of NaCl, K^+, and Ca^{2+} as well as causes reduced ability to concentrate and dilute urine [1]. A natriuretic, on the other hand, is any substance that promotes sodium excretion by the kidney [2,3]. Both loop diuresis and natriuresis result in increased volume of urine production due to inability of the kidney to concentrate urine [4–6].

Under apparently normal health condition, the glomerular filtration barrier (GFB) of the kidney provides efficient resistance to the passage of plasma protein into the Bowman's space [7,8]. Although some trace amounts of plasma protein can be detected in the urine under physiological conditions [7], the appearance of urine protein in large amount (proteinuria) is an important index of kidney injury or disease [7–9]. This makes proteinuria a health condition of clinical relevance. In humans, as determined by the integrity of the GFB, proteinuria can either be physiological or pathological [8]. While pathological proteinuria is associated with defective GFB, physiological proteinuria is associated with some conditions of the biological system such as ingestion of protein-rich

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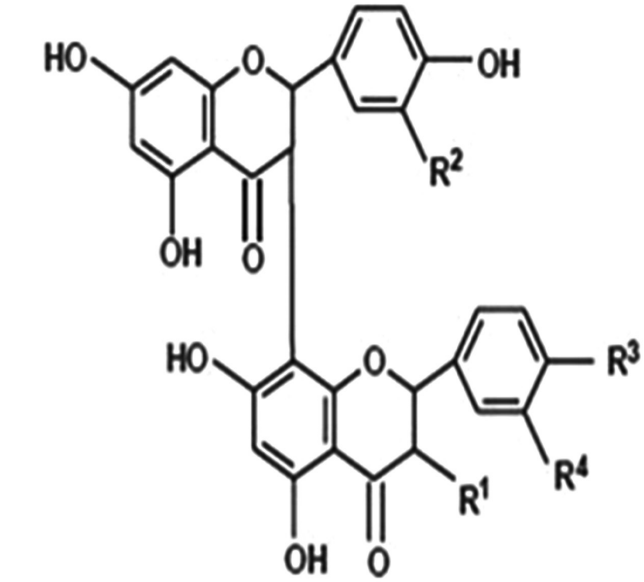
diet, vigorous exercise, pregnancy, hypoxia, fever, and posture [10]. Normally, unlike pathological proteinuria, physiological proteinuria is abated as these biological conditions are reversed or normalized. Contrariwise, in the absence of these biological conditions, apparently normal Wistar rats tend to excrete large amount of protein in their urine [8,11,12]. The experimental evaluation of this paradox in Wistar rats can point toward better prognostic and therapeutic approach against the life-threatening effects of proteinuria, an increasing incidence that is becoming a world health problem.

Garcinia kola seed, a species of flowering plant in the Clusiaceae family, is often called "bitter kola" because of its bitter taste. It contains an active phytochemical constituent "kolaviron" [13,14] (Fig. 1), which is an important biflavonoid existing as GB1, GB2, and kolaflavanone in the ratio 2:2:1 [13]. Kolaviron has been reported in the literature to exert therapeutic effects, basically, through anti-genotoxic [15], anti-inflammatory, [16] and antioxidant [17,18] mechanisms. Despite available literature on its vast medicinal benefits, this is the first (scientific) report on its effects in a model of proteinuria.



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OCH_3	OH

Figure 1. The chemical structure of the kolaviron.

Materials and Methods

Biochemical kits, chemicals, and metabolic cage

The kits for the assay of renal function biomarkers were purchased from Randox Lab. Ltd. (UK), while those required for electrolyte assays were purchased from TECO diagnostics company, Lakeview Avenue (CA, USA). The procedures for these assays were carried out according to the manufacturer's manual.

The chemicals used (n-hexane, methanol, and chloroform) for the isolation of kolaviron from *Garcinia kola* seeds were of analytical grade, available commercially. Propylene glycol (the vehicle used for kolaviron) was procured from Biovision (CA, USA).

The metabolic cages were fabricated by the Central Technological Laboratory and Workshop of the Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria [8,19].

Plant material and isolation of kolaviron

The seeds of *Garcinia kola* were purchased from a commercial vendor at Oja in Ikere Ekiti, Nigeria. This was, thereafter, certified by a Taxonomist in the Department of Botany, OAU, Ile-Ife with the voucher number IFE-17540. Kolaviron was isolated as described by the method of Farombi *et al.* [14], providing 96% purity of the isolated kolaviron. Briefly, *Garcinia kola* seeds were sliced, air-dried, and pulverized. Thereafter, the pulverized seeds were defatted using n-hexane in a Soxhlet extractor for 24 hours. The resulting dried marc was repacked and further concentrated using methanol. Thereafter, the concentrated methanol extract was fractionated using chloroform and the resulting yield was a golden yellow semi-solid substance called kolaviron.

Stock solutions of kolaviron

The adopted doses of kolaviron, as used in this study, were guided by its established oral LD_{50}. Kolaviron has an oral LD_{50} > 5,000 mg/kg [20]. The doses of the test-compound (kolaviron) were taken to be ≤10% of its LD50 [19,21–23]. Therefore, graded doses of kolaviron at 100, 200, and 400 mg/kg (p.o.) were adopted for this study.

The stock solution for each dose was prepared such that each rat received 0.2 ml of the solution per 100-g body weight. This was aimed at preventing any possible inherent adverse effect(s) of fluid overload. Therefore, the stock solutions for 100, 200, and 400 mg/kg of kolaviron were prepared by

dissolving 1, 2, and 4 g of kolaviron, respectively, each in 20 ml of propylene glycol.

Experimental protocol and animal management

The experimental protocol was made to comply with the NIH Guidelines for the care and use of Laboratory Animals [24] and approved by local Institutional Research Committee.

This study recruited 20 male Wistar rats, weighing 120–150 g. The study was carried out in the Animal Holdings Unit of the College of Health Sciences, OAU, Ile-Ife, where the animals were purchased. Each rat, housed in a separate metabolic cage, was allowed *ad libitum* access to standard laboratory rat feed under natural light and dark cycle. The rats were divided into four groups of five rats each. Group 1 orally received 2 ml/kg of propylene glycol for 30 consecutive days before they were sacrificed. Groups 2, 3, and 4 received graded doses of kolaviron at 100, 200, and 400 mg/kg (p.o.), respectively, for 30 consecutive days before they were also sacrificed. The rats were euthanized under ketamine anesthesia (60 mg/kg i.m.) and their blood samples were collected by cardiac puncture into separate lithium heparinized bottles. Thereafter, the blood samples were spinned at 4,000 rpm for 15 minutes using a cold centrifuge (Model 8881, Centrium Scientific) at −4°C. The plasma for each sample was decanted into separate plain (universal) bottles with the aid of sterile syringes. Each excised kidney was weighed and fixed in 10% formal-saline solution for histological examination using Hematoxylin and Eosin (H&E) staining technique.

Measurement of body weight, percentage weight change, kidney weight, and relative kidney weight

The body weight of each rat was measured weekly using Hanson digital weighing balance (Hanson, China), while the organ weight (at the point of sacrifice) was determined with the aid of Camry sensitive weighing balance (Camry, China). The following formulae were used to determine the percentage weight change and relative kidney weight [19,21,25]:

$$\text{Percentage Weight Change (\%)} = \frac{(\text{Weight of left kidney} + \text{Weight of right kidney})\text{g}}{\text{Initial body weight (g)}} \times 100$$

and [19,23]

$$\text{Relative Kidney Weight (\%)} = \frac{(\text{Weight of left kidney} + \text{Weight of right kidney})\text{g}}{\text{Final body weight (g)}} \times 100$$

Measurement of percentage changes in food consumption, water intake, and urine volume

Using metabolic cages, the food consumption, water intake, and urine volume for each rat were determined. While food consumption was determined with the aid of a weighing balance (Hanson, China), both water intake and urine volume were read off directly in a measuring cylinder. However, an insulin syringe was used to measure urine samples that were less than 1 ml [19].

$$\text{Percentage changes in food consumption, water intake and urine volume (\%)} = \frac{\text{Final amount (at the end of the study)} - \text{Initial amount (Baseline)}}{\text{Initial amount (Baseline)}} \times 100$$

Assay for electrolytes, renal function biomarkers, and determination of creatinine clearance.

TECO standard diagnostic kits were used to assay for both plasma and urine level of Na^+, K^+, and Cl^-, while Randox standard laboratory kits were used to determine both plasma and urine level of creatinine, urea, and glucose. The protocols for these assays were carried out according to the manufacturer's instructions. However, the level of total protein was determined as described by Lowry et al. [26].

Creatinine clearance was determined by the following conventional formula:

$$\text{Creatinine clearance (ml / minute)} = \frac{Ucv}{Pc}$$

where Uc = concentration of creatinine in the urine;
V = urine flow rate = urine volume/time (minute); and
Pc = concentration of creatinine in the plasma [8,19].

Note: Urine flow rate was determined by dividing the amount of urine collected over a 24-hour period (before the rats were euthanized) by the time taken to collect such an amount (24 hours = 1,440 minutes).

Determination of fractional excretion of Na^+ (FE_{Na^+}) and fractional excretion of urea (FE_{urea})

Both FE_{Na^+} and FE_{urea} were determined by the following standard formulae:

$$\text{Fractional excretion of sodium (\%)} = \frac{\text{UNa} \times \text{PCr}}{\text{PNa} \times \text{UCr}} \times 100$$

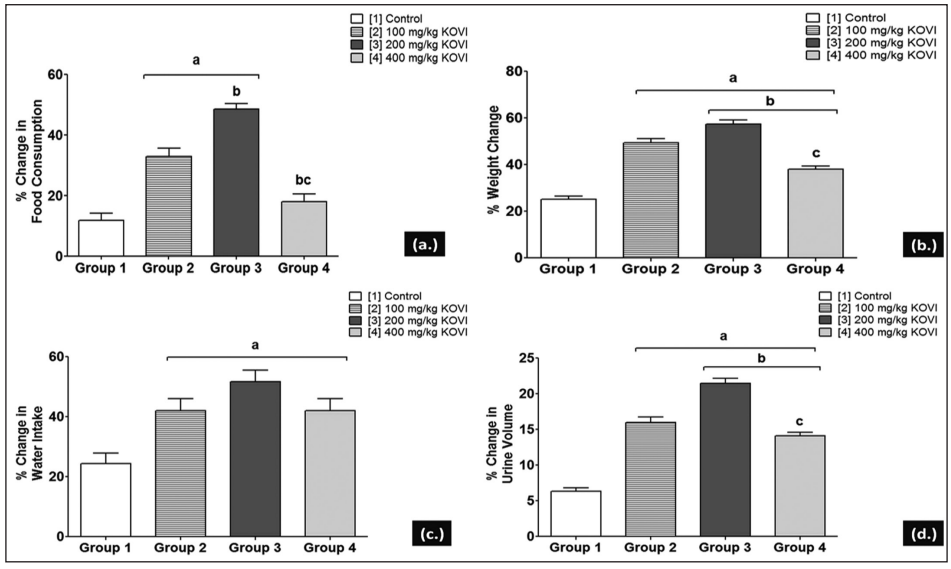


Figure 2. Effects of kolaviron on food consumption, body weight, water intake, and urine output of Wistar rats. Each bar represents mean ± SEM. [a]Significantly different from group 1 (control) at $p < 0.05$; [b]significantly different from group 2 (100 mg/kg kolaviron) at $p < 0.05$; and [c]significantly different from group 3 (200 mg/kg kolaviron) at $p < 0.05$.

where UNa = Urine sodium level, PNa = plasma sodium level, UCr = urine creatinine concentration, and PCr = plasma creatinine concentration [27,28].

$$\text{Fractional excretion of urea}\,(\%) = \frac{U\,\text{urea} \times \text{PCr}}{P\,\text{urea} \times \text{UCr}} \times 100$$

where *U*urea = urine urea concentration, *P*urea = plasma urea concentration, PCr = plasma creatinine concentration, and UCr = urine creatinine concentration [27].

Assessment of urine total protein and urine total protein–creatinine ratio

The urine total protein of the rats was determined using laboratory protocol as described by Lowry et al. [26].

For the conversion of S.I. units from dl/ml to mg/g in the determination of urine total protein–creatinine ratio, the following system of conversion was used [8]:

1 dl = 100g.

Histological examination

Each excised kidney was fixed in 10% formal-saline solution. This was, thereafter, dehydrated in graded alcohol before embedding in paraffin wax. About 6–8 µm sections were stained with H&E technique for histological examination using a Leica DM 750 camera microscope at ×400 magnification.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM), analyzed using one-way analysis of variance and, thereafter, subjected to Student's Newman Keuls *post-hoc* test at a level of significance $p < 0.05$. The statistical package used was graph pad prism 5.03 (Graph Pad Software Inc., CA, USA).

Results

Effects of kolaviron on percentage weight change and relative kidney weight of Wistar rats

Following oral kolaviron administration, the percentage weight change (PWC) was significantly higher in the kolaviron-treated groups 2, 3, and 4 when compared with the control (group 1) ($p < 0.0001$). However, group 4 (400 mg/kg) showed the least PWC, while group 3 (200 mg/kg) showed the highest PWC when comparisons were made between the kolaviron-treated groups ($p < 0.05$) (Fig. 2).

At the end of the study, no significant difference was recorded in the relative kidney weight of the kolaviron-treated groups when compared with the control ($p > 0.05$). The same is true when comparisons were made between the kolaviron-treated groups ($p > 0.05$) (Fig. 3).

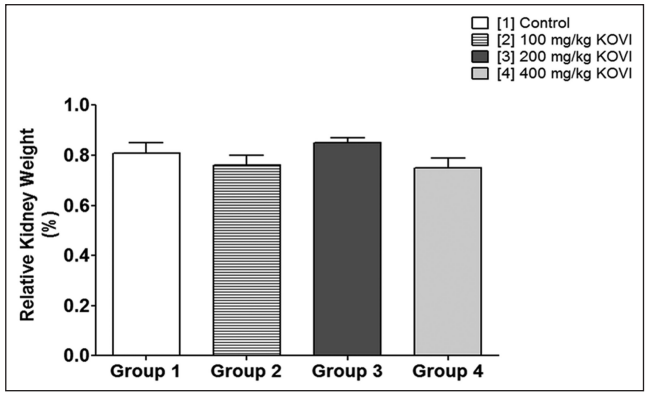


Figure 3. Effects of kolaviron on the relative kidney weight of Wistar rats. Each bar represents mean ± standard error of mean.

Effects of kolaviron on percentage change in food consumption, percentage change in water intake, and percentage change in urine volume of Wistar rats

Oral kolaviron administration was associated with a significantly higher percentage change in food consumption (PCFC) in the kolaviron-treated groups when compared with the control ($p < 0.05$). While group 4 (highest dose) showed the least PCFC, the medium dose (group 3) showed the highest PCFC, with a significant difference (non-dose dependent) when comparisons were made between the kolaviron-treated groups ($p < 0.05$) (Fig. 2).

Although no significant difference in percentage change in water intake (PCWI) was shown between the kolaviron-treated groups ($p > 0.05$), they had a significantly higher PCWI when compared with the control ($p < 0.05$) (Fig. 2).

The percentage change in urine volume (PCUV) of the kolaviron-treated groups was significantly higher when compared with the control ($p < 0.05$). The highest PCUV was expressed in the group 2 (medium dose), while group 4 (highest dose) showed the least PCUV ($p < 0.05$) (Fig. 2).

Effects of kolaviron on plasma and urine concentration of creatinine (mg/dl), urea [(plasma = mg/dl; urine = g/l)], and glucose (mg/dl) of Wistar rats

A significantly lowered plasma concentration of creatinine was associated with oral kolaviron administration in the kolaviron-treated groups when compared with the control ($p < 0.05$). However, group 2 (medium dose) showed the most lowered plasma creatinine concentration, while group 4 (highest dose) had the least lowered plasma creatinine level when comparisons were made between the kolaviron-treated groups ($p < 0.05$). This record is consistent with that of plasma glucose concentration ($p < 0.05$) (Table 1).

The plasma urea concentration was significantly lowered only in groups 3 and 4 when compared with the control ($p < 0.05$). However, group 4 (highest dose) had the highest plasma urea concentration when compared with both groups 1 and 2 (lowest and medium dose, respectively) ($p < 0.05$) (Table 1).

The urine creatinine concentration of the kolaviron-treated groups was significantly higher, following oral kolaviron administration, when compared with the control ($p < 0.05$). However, the medium dose group had the most significant urine creatinine concentration, with the highest dose having the least increase in urine creatinine concentration when comparisons were made between the treated groups ($p < 0.05$) (Table 1).

While no significant difference was recorded in the urine urea concentration of the rats when comparisons were made both against the control group and between the kolaviron-treated groups ($p > 0.05$), the urine glucose level of the rats was significantly lowered in the treated groups 3 (medium dose) when compared with the control ($p < 0.05$). Oral kolaviron administration was associated with a non-significant decrease in the urine glucose concentration of groups 2 and 4 when compared with the control ($p > 0.05$) (Table 1).

Effects of kolaviron on plasma and urine level of Na^+ (mmol/l), K^+ (mmol/l), and Cl^- (mmol/l) of Wistar rats

Oral kolaviron administration was associated with no significant difference in the plasma level of Na^+, K^+, and Cl^- when comparisons were made both against the control and between the kolaviron-treated groups ($p > 0.05$) (Table 2).

On the other hand, both urine Na^+ and Cl^- levels were significantly higher in the kolaviron-treated groups 2 and 3 when compared with the control ($p < 0.05$). No significant difference in urine K^+ was recorded in group 3 (medium dose) when compared with the control ($p > 0.05$) (Table 2).

Effects of kolaviron on creatinine clearance ($\times 10^{-3}$ ml/minute), fractional excretion of sodium (%), and fractional excretion of urea (%) of Wistar rats

While the kolaviron-treated groups 2 and 4 showed a non-significant elevation of creatinine clearance ($p > 0.05$), this was significantly higher in group 3

Table 1. Effects of kolaviron on renal function biomarkers of Wistar rats.

	Plasma concentration			Urine concentration		
Groups (*n* = 5)	**Creatinine (mg/dl)**	**Urea (mg/dl)**	**Glucose (mg/dl)**	**Creatinine (mg/dl)**	**Urea (g/l)**	**Glucose (mg/dl)**
[1] Control	0.76 ± 0.07	66.91 ± 6.92	92.06 ± 11.24	21.30 ± 3.95	212.50 ± 25.14	21.58 ± 2.00
[2] 100 mg/kg KOVI	0.36 ± 0.03[a]	54.57 ± 3.14	70.79 ± 5.07[a]	49.30 ± 5.29[a]	201.50 ± 19.91	16.79 ± 1.14
[3] 200 mg/kg KOVI	0.24 ± 0.08[a]	45.34 ± 6.90[a]	62.20 ± 4.53[a]	55.95 ± 6.01[a]	218.10 ± 16.14	14.21 ± 1.00[a]
[4] 400 mg/kg KOVI	0.72 ± 0.04[bc]	75.25 ± 2.26[bc]	91.32 ± 3.81[bc]	23.20 ± 3.41[bc]	165.70 ± 22.19	20.24 ± 1.31[c]

Each value represents mean ± SEM.
[a]Significantly different from control group at $p < 0.05$; [b]significantly different from 100 mg/kg KOVI group at $p < 0.05$; [c]significantly different from 200 mg/kg KOVI group at $p < 0.05$.

Table 2. Effects of kolaviron on electrolyte level in the plasma and urine of Wistar rats.

	Plasma concentration			Urine concentration		
Groups (*n* = 5)	**Na^+ (mmol/l)**	**K^+ (mmol/l)**	**Cl^- (mmol/l)**	**Na^+ (mmol/l)**	**K^+ (mmol/l)**	**Cl^- (mmol/l)**
[1] Control	136.80 ± 0.80	4.23 ± 0.23	69.76 ± 2.83	123.20 ± 1.16	137.60 ± 2.36	123.20 ± 1.16
[2] 100 mg/kg KOVI	136.60 ± 1.03	4.06 ± 0.15	70.40 ± 3.19	147.40 ± 1.66[a]	147.60 ± 2.03[a]	147.40 ± 2.66[a]
[3] 200 mg/kg KOVI	135.00 ± 1.05	4.03 ± 0.10	68.98 ± 2.16	140.20 ± 1.83[ab]	136.00 ± 2.21[b]	140.20 ± 2.63[ab]
[4] 400 mg/kg KOVI	136.60 ± 1.04	4.52 ± 0.10	69.00 ± 2.77	151.00 ± 1.34[bc]	149.50 ± 2.05[ac]	151.00 ± 2.34[bc]

Each value represents mean ± SEM.
[a]Significantly different from control group at $p < 0.05$; [b]significantly different from 100 mg/kg KOVI group at $p < 0.05$; and [c]significantly different from 200 mg/kg KOVI group at $p < 0.05$.

Table 3. Effects of kolaviron on creatinine clearance, fractional excretion of Na^+ (FE_{Na^+}) and fractional excretion of urea (FE_{urea}) in Wistar rats.

Groups (*n* = 5)	**Creatinine clearance (x10^{-3}ml/min)**	**FE_{Na^+}(%)**	**FE_{urea} (%)**
[1] Control	3.33 ± 0.50	3.21 ± 0.09	11.33 ± 0.25
[2] 100 mg/kg KOVI	4.20 ± 0.40	0.78 ± 0.07[a]	2.70 ± 0.15[a]
[3] 200 mg/kg KOVI	5.20 ± 0.50[a]	0.44 ± 0.06[ab]	2.06 ± 0.11[ab]
[4] 400 mg/kg KOVI	3.80 ± 0.30	3.43 ± 0.10[bc]	6.83 ± 0.19[abc]

Each value represents mean ± SEM.
[a]Significantly different from control group at $p < 0.05$; [b]significantly different from 100 mg/kg KOVI group at $p < 0.05$; and [c]significantly different from 200 mg/kg KOVI group at $p < 0.05$.

(medium dose of kolaviron) when compared with the control ($p < 0.05$). However, no significant difference in creatinine clearance was recorded between the kolaviron-treated groups ($p > 0.05$) (Table 3).

The FE_{Na^+} was significantly lowered in the kolaviron-treated groups 2 and 3 when compared with the control ($p < 0.05$). Between the kolaviron-treated groups, group 3 (medium dose) expressed the highest decrease in FE_{Na^+}, while group 4 (highest dose) showed the least decrease in FE_{Na^+} ($p < 0.05$) (Table 3).

There was a significantly lower FEurea in the kolaviron-treated groups 2, 3, and 4 when compared with the control ($p < 0.05$). However, group 3 (medium dose) expressed the highest decrease in FEurea, while group 4 (highest dose) showed the least decrease in FEurea when comparisons were made between the kolaviron-treated groups ($p < 0.05$) (Table 3).

Effects of kolaviron on urine total protein (mg/ml) and urine total protein–creatinine ratio (mg/g) of Wistar rats

The kolaviron-treated groups 2, 3, and 4 had a significantly lowered urine total protein concentration when compared with the control ($p < 0.0001$). The percentage difference in urine total protein levels was a decrease >80% in the kolaviron-treated groups when compared with the control (Fig. 4).

The urine total protein–creatinine ratio was significantly lowered in the kolaviron-treated groups 2, 3, and 4 when compared with the control ($p < 0.0001$). Also, the percentage difference in urine total protein–creatinine ratio in the treated rats

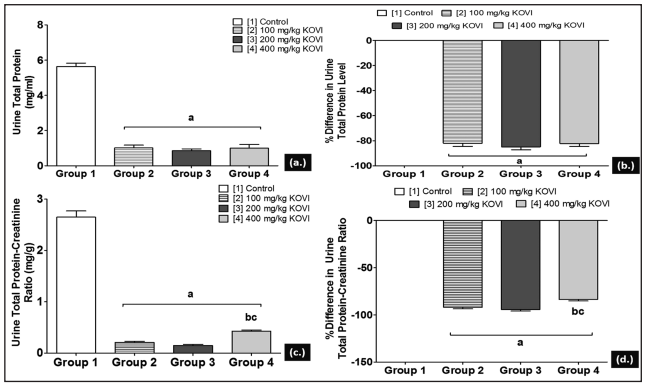


Figure 4. Effects of kolaviron on urine total protein and urine total protein–creatinine ratio of Wistar rats. Each bar represents mean ± SEM. [a]Significantly different from group 1 (control) at $p < 0.05$; [b]significantly different from group 2 (100 mg/kg kolaviron) at $p < 0.05$; and [c]significantly different from group 3 (200 mg/kg kolaviron) at $p < 0.05$.

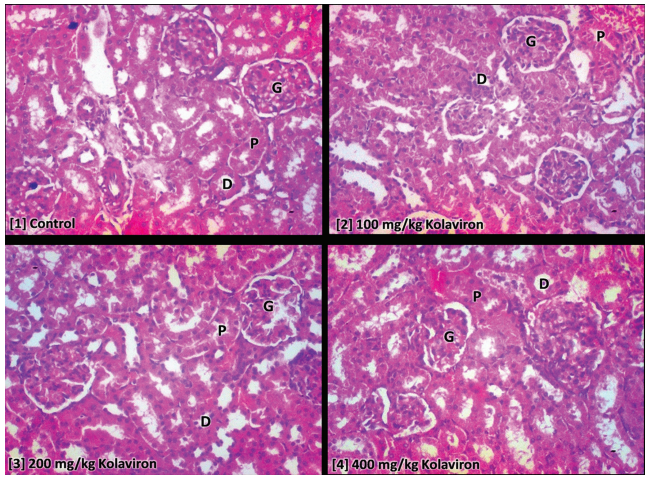


Figure 5. Histological effects of kolaviron on the kidney of Wistar rats. G = glomerulus, P = proximal convoluted tubule, D = distal convoluted tubule, [1] = group 1, [2] = group 2, [3] = group 3, and [4] = group 4. *Note*: There was no apparent histological change in the kidney of the rats following graded doses (100, 200, and 400 mg/kg) of kolaviron administration.

was a decrease of >80% when compared with the control (Fig. 4).

Histological effects of kolaviron on the kidney of Wistar rats

The histological examination of the kidney showed that oral kolaviron administration was associated with apparently intact histoarchitecture. Generally, the micrographic evidence showed no apparent distortion of the kidney features (Fig. 5).

Discussion

This study determined the renal effects of oral kolaviron administration in Wistar rats with physiological proteinuria. This was aimed at assessing the pharmacological activity of kolaviron on proteinuria using Wistar rat model. This was carried out by scientifically exploring the paradox of apparently healthy Wistar rats excreting protein in their urine. The study demonstrated that kolaviron mitigated proteinuria in Wistar rats. Generally, this biflavonoid (kolaviron) improved renal blood flow (characterized by the creatinine clearance) as well as sustained or improved the kidney histoarchitecture as depicted by the micrographic evidence.

Since relative organ weight can be an important index of the toxic effects of a chemical compound even in the absence of any obvious macroscopic change(s) [29–31], the sustained relative kidney weight that was shown to be within physiological range following oral kolaviron administration demonstrates an important health beneficial effect of this bioflavonoid in relation to kidney function.

The literature exists on the anti-diabetic potential of kolaviron [32,33]. This study, therefore, provides a supporting documentation of the hypoglycemic effect of this biflavonoid with its associated anti-glycosuric effect, thereby making it a potential therapeutic choice in the treatment or management of diabetes and hyperglycemic-related disorders.

The variations in the urine electrolyte levels that accompanied sustained plasma levels are indicative of a possible modulatory effect of kolaviron on the renal tubulo-glomerular feedback system. Although the sustenance of homeostatic level of plasma electrolytes can be partly attributed to the beneficial effects of kolaviron on feeding pattern, this study suggests that kolaviron apparently caused a modulation of the tubulo-glomerular feedback mechanism by the "inhibition of Na^+-K^+-$2Cl^-$ co-transporter in the macula densa to bring about reduced sensitivity (or insensitivity) of the juxtaglomerula apparatus to the circulating NaCl in the terminal end of the thick ascending limb" and (or) "inhibition of adenosine A1 receptors on the afferent arteriole, causing unresponsiveness to (possible available) adenosine, thereby promoting afferent arteriolar vasodilatation." Evidence of a possible enhanced afferent arteriolar vasodilatation was buttressed by increased renal blood flow that was characterized by creatinine clearance. These mechanisms present kolaviron as a loop diuretic. These features were associated with

increased urine output, as demonstrated by this study. Furthermore, the natriuretic potential of kolaviron (associated increase in urine Na^+ excretion) makes it a potential therapeutic choice in the adjuvant therapy for hypernatremia and hypertension which are usually associated with excess blood volume. Corroborating the suitability of this biflavonoid in adjuvant therapy for hypertension is its pharmacological activity of increased urine K^+ excretion. Usually, the risk of developing hypertension increases with low urine K^+ excretion [31,34].

A previous study, on Wistar rats, showed that increased urine protein excretion is directly proportional to the size of the GFB as well as the spaces between them (pores/slit pores); a phenomenon that is sex-dependent [8]. This study, therefore, observed a possible modulatory role of kolaviron on the GFB to bring about minimal plasma protein filtration into the glomerular ultra-filtrate. Another possible mechanism for the observed kolaviron-induced mitigation of proteinuria, subject to further scientific verification, is reduced synthesis of plasma protein by the liver and or increased tubular reabsorption of protein from the glomerular ultrafiltrate. Furthermore, since loss of negative charges around the glomerular basement membranes is also associated with the glomerular filtration of protein into the glomerular ultra-filtrate [30,35], there may have been a kolaviron-enhanced retention or accumulation of negative charges around the basement membrane resulting in a consequent repelling of protein (which are also negatively charged) since like-charge repels. On the other hand, the lowered urine protein–creatinine ratio of the rats, a clinical index for diagnosing non-benign proteinuria [36,37], as recorded in this study, points toward the fact that the biflavonoid potentiates sustenance of normal kidney homeostasis since a higher than normal urine protein–creatinine ratio represents a declining renal function [37].

The reduced level of both fractional excretion of sodium ion and urea (FE_{Na^+} and FE_{urea}, respectively) is indicative of the pre-renal effects of kolaviron, possibly on the modulation of urine protein excretion. Apparently, the reduced FE_{Na^+} and FE_{urea} were not consequences of decreased renal perfusion, as shown by the kolaviron-enhanced increase in creatinine clearance (an index of increased renal blood flow), rather, it may have been a modulatory effect of this biflavonoid on the tubulo-glomerular feedback mechanism, as aforementioned. Since FE_{urea}, unlike FE_{Na^+}, is dependent on passive forces and is less likely to be influenced by diuretic agents [38–40] or a potential diuretic agent such as the test-compound (kolaviron), the consistency in the pattern of these indices presents scientific facts that support the aforementioned proposed mechanism of reduced protein synthesis by the liver (pre-renal effect on the renal handling of substances) in an attempt to mitigate proteinuria.

Worthy of note is the fact that the highest dose of kolaviron (400 mg/kg) had the least pharmacological activity in almost all the assayed parameters. Apparently, this represents a note of caution indicating that high doses of kolaviron may be associated with derangement in renal function biomarkers, and possibly other biological indices, from physiological ranges. A further scientific exploration of this biflavonoid's mitigating effects on proteinuria is highly recommended. This should also consider its pharmacological activities in models of renal pathology. In additional, since this biflavonoid elicited both mitigation of proteinuria and the potentiation of loop diuresis, the relationship between these two pharmacological activities is worthy of further investigation to determine whether they are either dependent or independent variables in relation to renal homeostasis.

Conclusion

It was concluded that the kolaviron mitigated proteinuria and potentiated loop diuresis in Wistar rats. These pharmacological effects present the extract as a potential choice in the adjuvant management or treatment of proteinuria-associated nephropathies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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REVIEW ARTICLE

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The remember regeneration therapy method: An overview of new therapy protocol to approach diseases

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ABSTRACT

Epigenetic mechanisms, which cannot be explained by genotypic changes, but reveal the phenotype formation differences of genes, take part in the whole cellular process throughout our life. Recent advances in today's rapidly evolving technology have shown that erroneous epigenetic regulations can contribute to serious biological consequences and diseases in human. Human is the smallest model of the universe with its memory, which is processed into DNA codes within the cell, which is the smallest building block. All the phenomena we see as causal relationships in the human body are a reflection of "balance and harmony" relationship. We can see the intersection point of the quadruplet body (human body, energy body, electrical body, and energy channels) as a homeostatic system that produces continuous "balance and harmony" for the coordination of the inheritance DNA. As known, therapeutic approaches that are called as holistic or complementary medicine mainly target to the causes underlying basis of the diseases by using natural therapeutic agents and methods. Thus, it is primarily aimed to protect physiological mechanisms of the body. Also, such therapeutic methods allow medicine to avoid possible side effects of the modern therapeutic approaches. Probably, these are benefits of holistic or complementary medicine methods. On the other hand, many complementary and alternative medicine methods are commonly available, reasonable, and regularly used in many countries. And, these therapies are maintained by practical evidence on protection and efficacy. A growing amount of scientific clinical studies nowadays maintain the usage of definite these therapies. The Remember Regeneration Therapy Method (RTM) is a holistic medicine that describes the anatomical and physiological aspects of physiopathological changes in quadruplet body structures (QBSs), which is unique treatment system where phytotherapy is at its center and integrated with traditional and complementary applications. In the RTM model, diseases are seen as the reflection of epigenetic changes in the phenotype resulting from the gene-environment mismatch. The treatment strategy is based on the recovery of health by essentially improving the deteriorating structures. Considering that many of the epigenetic changes that cause disease can potentially be reversed, it has been clinically observed that epigenetic changes and irregularities improved when appropriate treatment protocols were applied, as in the RTM model. In conclusion, the main aim of this review is to introduce a new model of diagnosis and treatment to medical literature and to demonstrate the efficacy of RTM, which has been characterized by clinical experience (nearly 130,000 individuals suffering from several disorders) over a long period (approximately 25 years) and the reviewing of previous scientific knowledge.

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Introduction

Experiences are very valuable if they are based on clinical trials, and using this, the principal mechanism of diseases can be identified and on which new arrangements of screening, prevention, diagnosis, or treatment of can be used to cure the diseases [1–3]. Patients who have been dealing with chronic diseases may prefer new treatment programs in cases where Modern Western Medicine does not give satisfactory results [4–6].

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It is more likely that these results are related to the philosophy of the modern medicine. Athought almost every disease include various and different pathological mechanism and structural problems, most of medications used in current treatment protocols target spesific symptoms and sign of the diseases. On the other words, this is to see a small piece of a picture rather than to see big picture. Perhaps, the most important problem of the modern medicine is this perception.

It is recognized that the environmental, cultural, and financial factors currently influencing the world by consolidating with matters such as efficacy, quality of life, care, access to health care, cost-effective, and safety [7]. Considering the progress made in the science in the last century and particularly in biology, genetics, and epigenetics in the last two decades, it can be seen that many developments that were once imagined can be easily applied today.

Not only medicine will be used to heal disorders but also the features of a healthy human personality due to the quick development of biomedical investigations [8].

Environmental determinants such as air and water pollution, tobacco smoke, UV radiation, which trigger inflammation, are presumably the main reason for cancer development by forming oncogenic mutations [9]. Understanding that chronic inflammation is very important in many diseases leads to new horizons in treatments. Therefore, determining the interaction between the immune system and inflammation can help discovering how disease processes develop and how chronic inflammation is activated [10].

To date, approximately 80 autoimmune diseases (ADs), which present in the difficult and complex situations and of which underlying causes lead to intolerance by the immune system have been described, and these ADs have affected 5%–10% of the population [11]. It may be related to an irregularity in the immune system, a special modification of an immune-related gene, or the irregular expression of isolated epitopes, or misfolded composition of endogenous or exogenous substance occurring in the cells. Therefore, that has guided to the introduction of various hypotheses to describe the ADs in the medical literature (See the Reference #11 for review). As a result, the same systems might have a separate impact on the susceptibility in the periphery, local environmental components (e.g., gut microbiota), and field of inflammation controlled on tissue regions in the ADs. ADs have common characteristics such as (i) appearing in in adulthood, (ii) genetic and epigenetic modifications, (iii) sex, (iv) relationship with infections, and (v) autoantibodies of non-organ-specific individuals [11].

In cardiovascular diseases accompanied by atherosclerotic complications, the effect of oxidative stress (OS) caused by excessive nutrition, insulin resistance, impaired glucose tolerance (IGT), and diabetes has been demonstrated [12,13]. In one study, in addition to determining pathogenic issues facilitating the emergence of insulin resistance, it has been pointed out that the transition from insulin resistance to diabetes enhances the cardiovascular jeopardy status of prediabetic and diabetic people by IGT [14]. Therefore, the OS hypothesis can help us know how various curative medications capable of reducing OS can prevent or postpone the opening of diabetes and cardiovascular disease [14]. The ADs correlated with psoriasis frequently have one alike cellular response model, indicating that primary signal pathways may play a role in the knowledge of every autoimmune reply. Although a large number of large genome-wide association studies exist, an exact causal locus for psoriasis or other ADs still remain unknown [15]. In recent years, clinical and scientific evidence from the therapeutic approaches to intestinal dysbiosis in inflammatory bowel diseases has strengthened the views of complementary and alternative medicine (CAM). New scientific researchers have shown that sensitive and new generation CAM strategies for the treatment of specific disease conditions will gain great advantages with improvements in research technologies [16].

Today, a number of CAM practices such as acupuncture, massage, light therapy, etc. have achieved quite satisfactory results in the treatment of patients. For example, the efficacy of acupuncture on traditional therapy for pain relief has been described by various clinical trials [17,18].

Although these metaphysical definitions are made while explaining the positive results from the pathophysiological point of view, the applications are officially legitimized in the whole world under the name of complementary and integrative medicine after long and troublesome years. However, it is more appropriate to explain the success of these treatments with epigenetic mechanisms by new developments in science, because there are no specific molecular and pathophysiologic target for these therapeutic approaches. Also, in these treatment approaches, the philosophy of holistic can make the adaptation of the body to new state

and curing the diseases resulting from the internal changes caused by factors and physiopathological processes.

Epigenetics is included in several natural cellular manners. In the human body, cells, tissues, and organs are modified by the "opening" or expression of certain gene groups, or the "closing" or suppression of other gene groups [19].

There are three methods within the cell that can interact with each other to silence the genes: DNA methylation, histone modifications, and RNA-related silencing [20].

Briefly, the Remember Regeneration Therapy Method (RTM) is a *holistic approach* that includes the combination of several complementary and traditional medical methods. Diseases are recognized as the reflection of epigenetic changes occurring as a result of gene-environment disparity to the phenotype in the RTM model, and the treatment strategy is mainly based on the recovery of human health by improving the deteriorated structures and functions.

The primary aim of this review is to examine the efficacy of RTM, which has been characterized by clinical experience over a long period and presents a new model of diagnosis and treatment.

Physiological condition

The body is a whole, and it is never a machine functioning alone in accordance with the system it creates. The biological, histological, and physical -structure of the human body is composed of living cells and extracellular materials, and there are a perfect network that provides connection and information exchange between each other. Autonomously, the human body is able to work hard to believe by sending the signals that pass through our brain at a very high speed every day [21].

Today's conception of medicine considers the body as a mechanical system that is independent of the mind and the spirit, which is not related to universal external factors. However, there are scientific studies that prove the impact of universal and environmental factors such as air and water pollution, UV radiation, and chemical exposure on the general health of the body. Modern Western Medicine does not refuse the work of the evidence itself, but still prefer to stay away from them [22]. However, as our knowledge about human being increases, a different complex structure that maintains the body structure with the soul can be revealed.

Cell and DNA

The human body is physically composed of the same basic material. In nature, all living creatures are composed from cells, some of which are formed of a single living cell and termed single-cell organisms. Therefore, all components of our body are building blocks of different cells with chemical and structural properties. In humans, there are approximately 200 various cell species and 20 various compositions or organelles [23]. DNA is a long chain molecule that performs an important function in our lives. The genetic structure of all organisms controls the information encoded in DNA strands. DNA basically performs a function in replication, gene expression, coding information, mutation, and recombination [24]. DNA is a constantly acting molecule; when a cell is ready to separate, it is transcribed and read to create molecules such as proteins to perform cell functions. DNA molecules in a unique human cell from one end to the other have a length of approximately 2 m, and the human genome is about 3 billion base pairs [25].

Human body and quantum theory

According to the quantum theory, the substance is not consisted of fixed concrete molds but has a variable structure that changes according to the actions taken from the meaning. Everything we see or perceive in our environment and body is the images of the vibrations generated by the subatomic structures in the energy dimension [26]. According to this prediction, the structures that vibrate in the energy dimension are shaped as visual objects in the body dimension. The universal knowledge-dimensional, which is from the "Universal Information Center (UIC)" thought revealed by Quantum, is the universal energy field, the universal vibrations, and the universal polarization, all say that everything is a partnership. This partnership, from our cells to the great design in the universe, shows that the whole universe is fed from a common source of information. As the visual formations depend on the energy signals, the transmission of the energy signals depends on the information under the signalization. Our body is a tool for detecting environmental signals throughout life.

The soul is what the beings who have DNA need to have life. Humans provide bodily form and functioning by transferring the information they have with universal knowledge to the body through their DNA [22].

Pitfalls in modern medicine

Nobody can discuss the importance of evidence-based medicine. It provides the allocation of informed determinations and limited resources to carefully measure the safety and effectiveness of the drugs and tools used to treat our patients. This practice continues to apply in dermatology as well as in other medical fields. In the evidence-based medicine (EBM), medical doctor's private information may be due to deliberate prejudices and the possibility of the emergence of a high degree of misconduct to reduce or even prevent [27].

Nowadays, the fascinating modern medicine relies on a quite conservative scientific strategy for symptoms, diagnosis, and treatment. This modern medicine is so limited that it can clearly disregard any alternative treatment and all other therapeutic practices. Critical intensive care is based only on the therapeutic procedure when life is on a limited range. In our knowledge, 21st-century faith is solely a convicted medical program. At an increasing level, we expect a medical drug such as a magic wand that can cause health problems to dissolve without having to alter the way we live [28].

EBM is often described as "conscious, open, and logical use of the best evidence currently available to decide on the responsibility of patients" [29]. EBM is not limited to randomized clinical trials and meta-analysis. It includes following up the excellent exterior confirmation to explain the clinical problems. We require to determine the correct cross-sectional investigations of presumed subjects with this clinically associated disease, not each randomized research [29]. It is hard to think that EBM can only encourage doctor–patient cooperation. Nevertheless, EBM is not far from the effects of pharmaceutical manufacturing. Critics believe that, in contrast to expectations, EBM allows physicians to intervene in the direction of directing and managing this relationship, in addition to improving doctor–patient cooperation. As the pharmaceutical business maintains its effectiveness on EBM, doctors will have to be more alert to treatment decisions by patients. In order to avoid such problems, doctors need to develop new ways of approaching and giving information to the patient [30]. Today, the EBM is not considered to be magnified in modern health services, because scientific interpretations, as an international health service model, are seen as the main way to be followed [31].

While the EBM-based curriculum is modifying medical training, evidence-based thought also led to a review of as well as nursing and health-related policies [32]. Most of the EBM questions naturally originate from medical fields. As well as, in a number of systematic questions about the production of robust guidelines when evidence is inadequate, they emphasize the devastating influences of measures at the regional level. In fact, the work is a kind of personal ability or art that can result in a much higher measure of subject responsibility which is permitted to mention personal competence and practice [32].

In order to overcome such problems, it is not mandatory to stay on the applications very much. However, there is a need to implement clear policies in order to make the best use of the common and relevant aspects of the work on medical matters. The versatile strategy to limited partners and their concerns is more logical than the policy of resolving the obstacle of clarity by minimizing expert independence [32].

Complementary and alternative therapy methods

In the United States and Canada, more than 70% of people use the CAM method at least once in their lives [33–35], which is thought to spend billions of dollars in these treatments [36]. CAM is a common definition traditionally used for health practices that are not part of conventional medicine. In fact, in many cases, as evidence of efficacy and safety increases, these treatments are combined with traditional medicine. Thus, rather than the definition of alternative medicine, more recent terms such as CAM have been used. Complementary medicine can help improve the quality of life by reducing the symptoms of cancer, persistent pain, chronic fatigue, fibromyalgia, and similar symptoms of many patients (e.g., pain and anxiety) [37]. CAM has been described as an application of "different medical and healthcare interventions, applications, products or disciplines" which are not accepted as part of standard medicine. The National Center for Complementary and Complementary Integrative Health (NCCIH) located at the National Institutes of Health (NIH) describes as "complementary" when non-mainstream applications are used in conjunction with conventional medicine, and "alternative" when conventional medicine is replaced by "complementary", and "integrative" when conventional and complementary methods are combined with the practice and coordinated program [38]. In a study, it was stated that CAM guidelines, which received a good score from various evaluations, can be be evaluated mainly by patients and health

specialists regarding the application and outcomes of CAM therapies [39].

Nowadays, most people who apply non-mainstream methods practice classical health service. However, instead of complementary and alternative, the word "functional medicine" is used. Integrative healthcare services frequently combine both conventional and complementary methods in an organized manner. The definition of health-care and wellness (usually mental, emotional, functional, spiritual, cultural characteristics) indicates a holistic, patient-oriented procedure that completely treats a complete individual rather than a body system. The NCCIH, located at the NIH, supports the organization of research to improve considerably critical scientific and public health care issues regarding Complementary and Integrative Health strategies. According to NCCIH data, more than 100 million Americans spent $600 billion on complementary health approaches. As indicated in the NCCIH's Strategic Plan, research on chronic pain, which causes cost and productivity loss and insufficient conventional medicine, is the main focus [40].

A conversation involving the approval of the opinions and choices of subjects and their relatives has the opportunity to develop the shared parts needed to improve healing. Supplies granted by NCCAM can support health personnel amplifies their information and experience of CAM applications [41].

The use of CAM is actually quite common and varies from country to country. Rates of CAM application are 20%–40% in the United Kingdom [42,43]. It is stated that CAM is used by cancer patients around 20%–77% by Patient–Physician Conversation [44].

There is a notable interest for CAM applications in the world. The World Health Organization (WHO) has stated that in developing countries, several CAM applications have been in the primary health care (PHC) service and that has become more widespread [6]. On the other hand, many economic and social reasons increase excited enthusiasm for CAM applications in industrially developed countries [6,45]. In a study, the main purpose for practicing the highest CAM methods among children with mental health concerns was found as the CAM's complementary function to disorders administration (combined with conventional therapy) [46].

Epigenetics

New and outstanding discoveries in the science of biology and genetic science over the past 25 years have shown that inheritance has a whole new dimension beyond the genes, not in the structure of the DNA, but in the study of the genes that there have been significant changes in life. In this new dimension of inheritance called epigenetics, it has been shown that changes can be transferred to new generations [47,48]. In addition, it is not surprising to see that epigenetic mechanism (examples of epigenetic memory that use relevant mechanisms over different time scales: cellular memory, transcriptional memory, and transgenerational memory) provide all organisms to adjust to environmental differences on long-term measures even for generations and an organism modifies to any incentive [49].

Current information on the significance of epigenetics in diseases promises an important prospect for epigenetic study. The performance of epigenetics in diseases has attracted more attention in describing complex diseases such as cancer, behavioral plasticity, and AD for a long time. Therefore, it has been hoped that the performance of the relationship of some epigenetic markers with special disorders may improve the instruments to be used in the diagnosis and prognosis of the disease [50]. The importance of epigenetic modifications in long-term memory performance has been demonstrated in the most extensive and comprehensive study on the significance of DNA methylation. Chromatin remodeling, histone modifications, and non-coding RNA mechanisms are other important changes. DNA methylation models are also known to produce an irregular representation of cancer-dependent genes [51]. In the future, the human epigenome project will solve the model of DNA methylation in various tissues and mediate the organization of gene composition is each chromatin or DNA, or both [52]. Consequently, it may be possible to identify the appearance forms of responsive genes sensitive to environmental effects, resulting in epigenetic characteristics for human disorders and environmental disclosure [53]. In addition, the hypothesis is proposed that hereditary epigenetic modifications in chromatin formation may perform a significant function in development. According to this pattern, an environmental incentive is capable of inducing hereditary chromatin changes, which may result in a highly particular, anticipated, and adaptive reply [54]. The significance of epigenetics in cancer is well known. And, it is expected that rapid developments will be experienced in this short time. Because of the progress in technology and new highly efficient systems has addressed reasonably to investigate the more extensive epigenetic manner even a single gene. Next-generation

sequencing methods provide the research of the DNA methylation mechanism situation of cells at the nucleotide level [55].

In light of today's information, epigenetics, defined as the adjustable regulation of gene silencing and expression without changing the gene DNA, can link special genes relating various environmental determinants related to ADs [11]. In a review of ADs, many hypotheses were discussed, and what is defined as the "nucleolus hypothesis" of ADs, could give a description for the female bias, the engagement of epigenetics, the source of most autoantigens, and the existence of specific mechanisms that are appropriate in various relevant ADs [11]. Consequently, as researchers progress towards epigenetics to find explanations to AD inquiries, the nucleolus hypothesis continues to keep important matters to be discussed [56].

In summary, DNA methylation is an epigenetic variation of DNA which has a significant role in the regular organization of transcription, embryonic development, imprinting, and genome stability [57]. DNA methylation models are interrupted in cancer, with genome- and gene-specific hypomethylation, and hypermethylation, proceedings happening concurrently in the similar product, respectively [57]. X inactivation is regulated by a complicated genetic locus termed the X-chromosome and is marked in extra-embryonic tissues of eutherian animals [58]. An interesting feature of imprinted genes in which they frequently gather in massive chromosomal regions, increasing the probability that gene- and domain-specific mechanisms manage imprinting [59]. On the other hand, the critical function of DNA methylation and the communication among different epigenetic administrative route not only contributes an understanding of cell differentiation, evolution, and functions related to the formation of diseases but also supports in the improvement of active stem cell-associated therapies and clinical beneficial compound inhibitors [60].

In the future, the function of methylation in transcriptional regulation, chromatin structure, DNA repair, and genome stability is expected to be the focal point of intensive and specific investigations in the area of DNA methylation in epigenetics. Thus, the characteristics of such communications are thought to shed light on the pathogenesis of disorders of methylation patterns and innovative therapeutic agents in cancer cells [61]. The primary origins of epigenetic mechanisms and their participation in human wellness, the use of epigenetic pathways in targeted therapeutics, novel strategies are constantly being developed. With the results of these studies, the risk of epigenetic disorder that leads to disease is reduced and in later life epigenetic errors are corrected. Thus, epigenetic treatments will be replaced as a new treatment option in medicine soon [62].

The importance of epigenetics has become more prominent with the increasing recognition of the role of specific epigenetic mechanisms in cancer which ensures to combine genetics and epigenetics [63]. Today, advances in science focus on DNA methylation, histone modification, non-coding RNAs, and changes in chromatin structure with the help of molecular-level high-scale technologies [64].

In addition, the life cycles of the cells, which are the basis of life, contain important clues to understand the pathophysiology of diseases. It is appreciated that the event of senescence, called cellular senescence reveals the nature of cellular ageing caused by telomere loss due to the lack of endogenous telomerase activity following great reproduction [65]. Basically, OS reactive oxygen species (ROS) causes epigenetic changes. Studies have shown that intracellular and external cellular stresses stimulate the cellular senescence schedule. These stresses initiate a change of cellular signaling processes, resulting in a DNA damage response. In particular, ROS is leading to DNA damage response by disrupting gene transcription and DNA replication in addition to telomere shortening. In cells exposed to severe stress, a mechanism that is not yet fully understood is activated and cellular senescence begins. Cells that undergo senescence may also initiate an inflammatory process, independent of senescence causing stress [66].

The effect of senescence and epigenetic changes caused by chronic toxicity and chronic inflammation on cellular and tissue levels will lead to the development of new approaches to diagnosis and treatment of many diseases.

Phytotherapy may be a different therapeutic approach. Studies have shown that some phytochemicals may change abnormal gene activation or silencing in addition to normal epigenetic events. It has been shown that the compounds in many food support products (teas, garlic, soy products, herbs, grapes and cruciferous vegetables) have anticarcinogenic effects as epigenetic modulators and can perform a character in the regulation of biological processes [67–69] (Fig. 1).

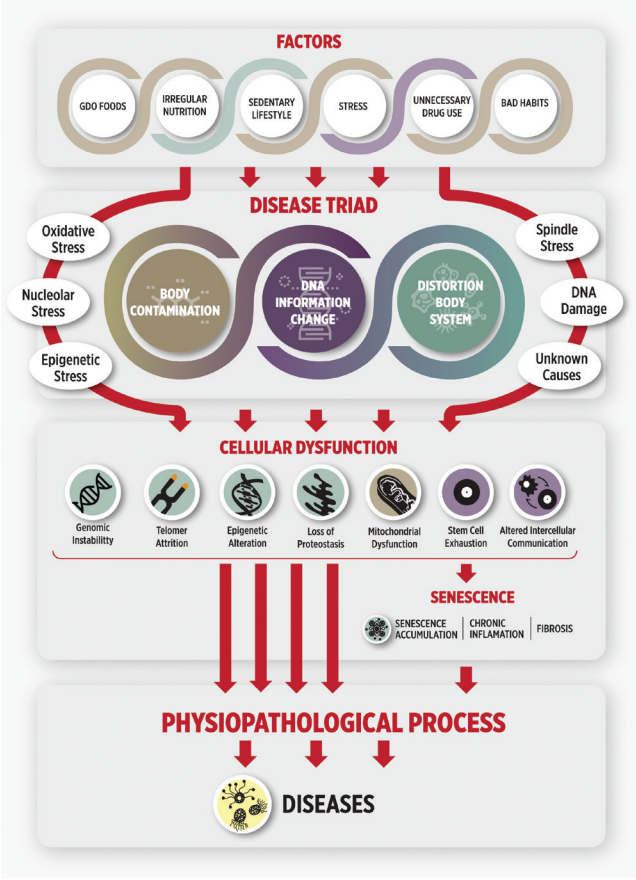

Figure 1. The molecular basis of the dieases and its inducers.

The remember regeneration therapy method

Under the light of the knowledge above mentioned, it may say that human organism has a complicated structure and different mechanisms, and in any case of disease, all of these may be cause pathological conditions by altering normal processes. Therefore, the treatment of any pathological conditions depends on the normalization of all altered process. But, for this normalization, a medication planning which aim to ameliorate all impaired physiological mechanisms should have been developed rather than a medication that targets a single pathologic mechanism. For instance, such medication may include just one phytotherapeutic agent, or a combination of various holistic medicine methods such as phytotherapy, ozone therapy, acupuncture, etc. In other words, it is important to determine the most ideal combination of holistic medicine methods in order to normalize impaired mechanisms, setpoints and even molecular structure such as DNA. So, the mechanism of effect of each treatment methods should be known and related to the pathophysiological mechanisms of detected diseases. Thus, the content of these treatment combination will vary according to various factors such as individual features of the patients, the severity and extent of the diseases. This treatment philosophy constitutes the basis of the RTM.

One of this pathological mechanisms which is targeted by RTM is also epigenetic alterations. It has been shown that epigenetic alterations may play important role in many diseases including cancer, and a number of bioactive dietary components forming the content of phytotheraphy placed in center of RTM also show beneficial effects due to epigenetic modifications [69,70].

RTM is a holistic approach to diagnostic and treatment system with phytotherapy and combination of several complementary and traditional medical methods such as acupuncture, cupping therapy, hirudotherapy, ozone therapy, etc. since the 2000s. The efficacy of RTM has been characterized by both clinical experience and observation (nearly 130,000 individuals suffering from several disorders) over a long period (approximately 25 years) by laboratory evidence.

The most important point here is the different point of view of the diseases and treatments. In the RTM system, diseases considered as adjustments of the body to new manners of structure, and these modifications are in fact positive new processes that the body has settled forward to sustain errors. In order to initiate the healing process, it is essential to eliminate the factors that push the body to these conditions and to correct the internal changes that occur in the body, and at the same time, convert the body to its former form in the new information and adaptations in the cellular DNA module. When we compile all of these, it can be summarized as the RTM [22].

Quadruplet body structure

QBS covers all structural, visual, and functional functions in our body originating from DNA. The functioning of DNA information is important and works together with all body structures. The whole aim is to sustain life and to achieve these setpoints should continuously be adjusted. There is harmony in the biological system. The alignment between the quadruplet structures is important in providing body setpoints. The aim of harmony and balance is the sustaining of life, and the maintenance of life is a requirement. Equilibrium relationship and harmony rather than causal are recognized as a disease. Biological systems are semi-autonomous (cell, tissue, organ, system, etc.), and there is a balance between existence and absence (semi-autonomous structure).

The most important information in DNA coordination is the functional information that provides the body activity. While standard body activities are coordinated with standard setpoints, reflex body activities are coordinated with reflex setpoints. Standard body activities are performed on the standard setpoints produced by the first information in DNA.

According to the RTM system, the human has four body structures (QBS). The first one is called the *human body* and represents the known physical structure of man. Although the *electrical body* is the second body structure used for diagnostic purposes, its use in treatment applications is limited. It is possible to see the functions of this body with diagnostic devices such as EEG, ECG, and EMG. Our third body structure is our *energy body* and covers the field of yoga and meditation. It is the body area where seven energy centers called the Chakra are located. In order to provide information exchange, the *energy channels* that form connections between the human body and the energy body can be defined as our fourth body structure and the movement area of the acupuncture treatment is related to this body structure.

The "universal knowledge" which from the "UIC" is based on the "Primary DNA Change" in the DNA. The pathogenic effect does not produce a change in DNA after a reflex response. If the pathological process continues, DNA changes in the body. The primary goal of the body is to provide vital values. Strong and persistent pathogens affect DNA and cause the "Secondary DNA Change" and then it causes a cell dysfunction. All of them are result in "Third Setpoints" and cellular structure change (e.g., hypertension, diabetes, elevated cholesterol, ADs, circulatory disorders, chronic disorders, etc.). The tables defined as the disease are actually new setting changes. Thus, the disease expression in the RTM module is defined as "new setting changes". Thus, three separate mechanisms are involved in the introduction of the new body values: (i) DNA information changes, (ii) Contamination due to factors, and (iii) Bodily changes.

There are many cells and structures in our body that confirm this knowledge. Merkel cells, which are one of them, are cells composed by solid secretory granules and cytoskeletal skin and in some mucous membranes. The conjunction of the Merkel cells to the brain is two-way, which not only receive but also has the ability to emit electromagnetic pulses. Therefore, the efferent perspectives of palmar and plantar Merkel nerve finishes constitute the bottom for biofield modalities. Merkel cells are multisensorial cells that can collect the whole surrounding environmental incentives, including electromagnetic waves. As a result, Merkel cells are highly functional cells that can meet in one point between conventional and complementary medicine [71] (Fig. 2).

Setpoints

Functional information that is vital in life is constantly dictated to our body by DNA. Our body also serves to perform the functional body values of all bodily activities from the DNA to the cells, from cells to tissues, from tissues to organs and systems. The information dictated by the DNA from our bodies creates fixed points that regulate the functional functioning. The body organizes its functioning according to these fixed points (setpoints). Standard set point activities are normal body activities that are demonstrated without active DNA support. As a result, set point values are created.

In order for the body to continue to function, the initial information from the DNA is sufficient. According to this information, setpoints are vital standards for us. The activities of the reflex body are also carried out depending on this information. It is the body and energy body togetherness that represent the substance and mana contacts which are the main components of the coordination of functional activities. When exposure to chronic pathogens and the existing functional parameters do not suffice to maintain life, the DNAs in both the substance realm and the soul are actively engaged to form new parameters (seconder setpoints) for

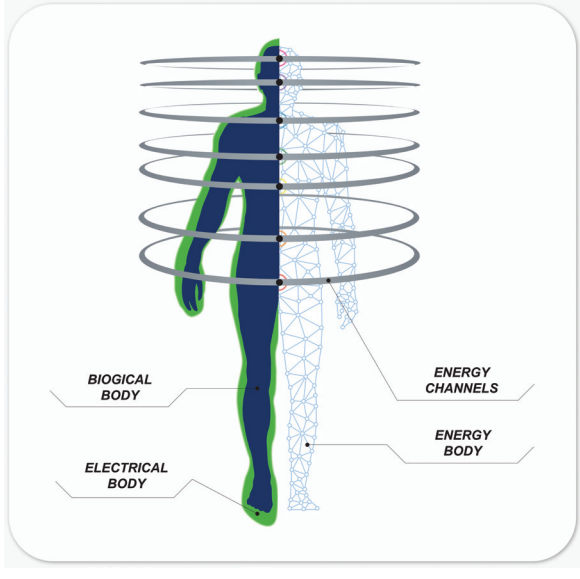

Figure 2. The components of the QBS.

the maintenance of life. The energy channels that is same with the "Meridians" defined in Traditional Chinese Medicine are special connections providing the exchange of the functional knowledge between the energy body and the human body according to the RTM method. Thus, the pathologies related to both kinds affect each other. It is a requirement of the two-body coexistence.

As an example of epigenetics in the formation of diseases, essential hypertension, the etiology of which is not very well known, is a complicated multifactorial disease with epigenetic and environmental determinants according to its currency. In human physiology, the sympathetic nervous system performs a significant function in the keeping of hypertension and the rostral ventrolateral medulla (RVLM), which is the principal cause of this sympathetic activation. A reasonable mechanism for explaining sympathetic hyperactivity in RVLM is the/a movement in the area postrema (AP). Thus, it seems likely that AP appears to be the area of high blood pressure, which occurs in hypertension. It has been suggested that epigenetic changes from melatonin in AP neurons has a task in this change and shift in AP (set point). According to this hypothesis, the AP has been described to include great levels of melatonin receptors that are involved in epigenetic changes in specific cells [72] (Fig. 3).

Diseases and new set points

Functional values alter according to the differences in the body. The new values are the *new setpoints* that are perceived as diseases. Three mechanisms are involved in the formation of new settings (setpoints) perceived as diseases in the body. These are called the *disease triad* in the RTM system and consist of three components: contamination of the body, system degradation, and DNA information change. The *treatment triad* is needed for treatment of diseases and return to primary settings, cleaning the body, regulating systems, and resetting DNA [22] (Fig. 4).

RTM treatment model

In the treatment triad, RTM module is mainly related to deteriorating structures after three mechanisms: (i) DNA information changes. DNA Information Process has no place among current

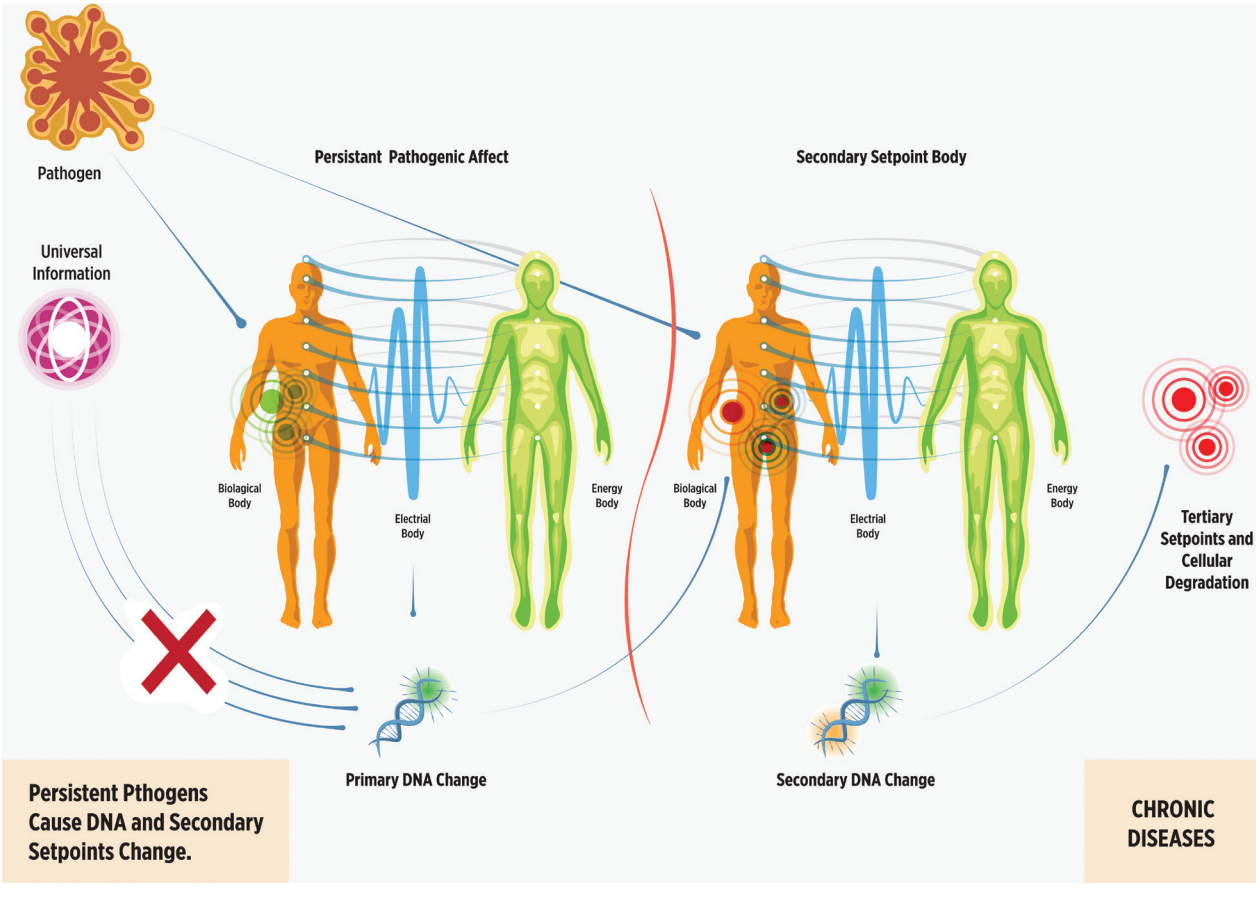

Figure 3. The changes of DNA and seconder setpoints in QBS.

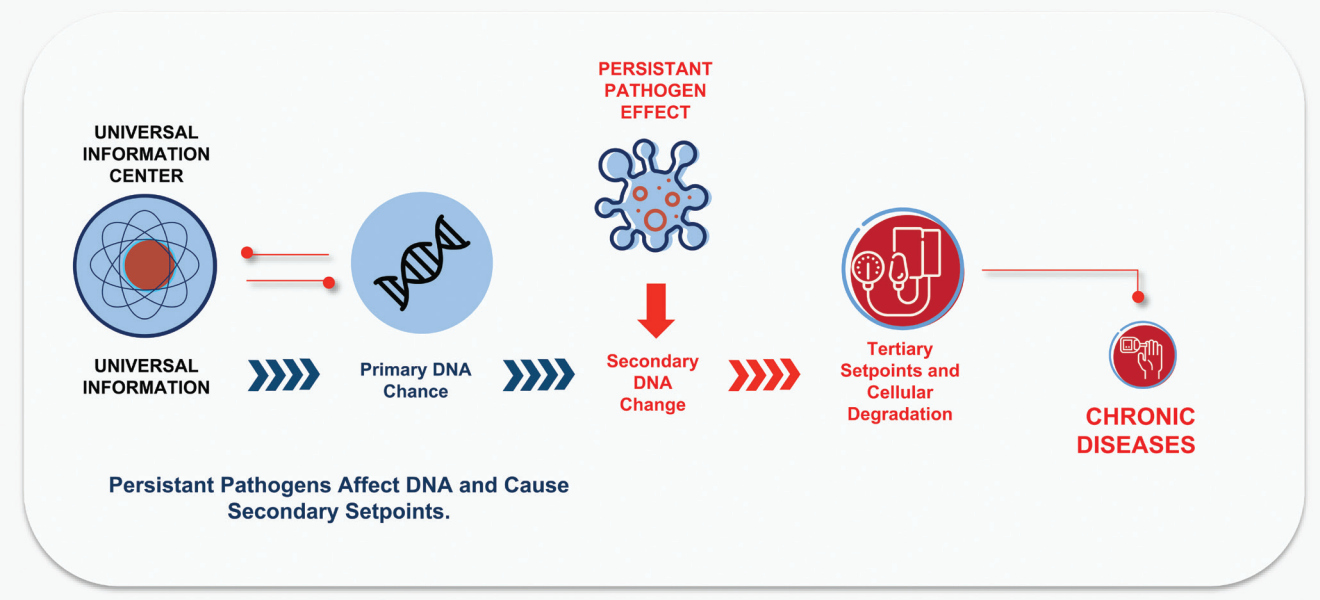

Figure 4. Chronic diseases due to interaction between universal information and DNA.

treatment methods and protocols. (ii) Clearing of physical contamination.

Every known treatment method tries to treat only its own Body Section. For example; cupping, ozone, colon-therapy, phytotherapy applications. (iii) Organization of systems. For this, the current treatment methods are used: acupuncture, reflexology, bioenergy, and phytotherapy applications.

Mechanisms

The mechanisms that is responsible for the efficacy of RTM can be classified under two main groups: *remember phase mechanisms* (in the resetting of DNA, the phenotypic structures in DNA are eliminated and the body is returned to the original information) and *regeneration phase mechanisms* (body cleaning application-detox and regulation of systems) [22].

In the remember phase, main mechanism is epigenetic regulation, and bioactive molecules used as phyto therapeutics are responsible for the normalization of epigenetic alterations [68].

In the regeneration phase consist of two sections: detox and the regulation of systems. The mechanism of action of the detox process is the reducing of free radicals and the increasing of antioxidants. Cupping and ozone therapy is some of the treatment methods used for this purpose [73,74].

The mechanisms of action played role in the system regulation phase is the regulation of impaired enzymatic and hormonal functions and the restoration of cellular communication with phytotherapeutic agents [75,76].

For the regeneration section, the mixtures are prepared from the leafy and opaque parts of the plants, while another mixtures consists of seed plants are used for the remember section. In the RTM treatment system, the lion's share in the success of the therapy belongs to the RTM therapeutics. In addition, the treatment is supported by various complementary medicine methods such as acupuncture, cupping, reflexology, hirudotherapy and ozone, etc. in order to accelerate the healing process. These CAM applications can be reproduced according to the condition of the patient and the disease. Briefly, RTM is a patient-specific treatment model that aims to treat the patient, not the disease [22].

Phytotherapy products

This approach is in RTM as, (i) Remember Phase: Seed plants for DNA resetting; (ii) the Regeneration Phase: The use of leafy plants for the cleaning of the body and the regulation of the systems. Phytotherapeutic products in the simplest form used are as follows: (i) Remember Phase and (ii) Regeneration Phase [22].

Follow-up and control

Time intervals are in the follow-up and control of patients as Weekly Close Follow-up, Monthly Follow-up, 40-Day Follow-up, and 3rd-40 Daily Follow-up.

Conclusion

In the RTM model, it is thought that epigenetic processes were wrongly developed and organized in diseases and contribute to phenotype of disease in human body. In the RTM Model, the treatment strategy is based on the recovery of health by essentially improving the deteriorating structures. Future studies should examine how mutations in genes that have modified epigenetics contribute to phenotype in diseases.

Importantly, many of these changes are potentially reversible and can be changed by treatment with appropriate drugs. Since epigenetic processes are at the root of biology, they have implications in all of human development and disease.

Our strategy in treatments has focused fundamentally on an original holistic approach, which we believe is based on interactions between processes such as DNA methylation, histone modification, and nucleosom setting to construct the epigenome. For this purpose, different treatment combinations consist of various holistic medicine methods such as acupuncture, ozone therapy, hirudoterapi as well as phytotherapy are determined. Also, these combinations vary from disease to disease or from person to person because each disease belongs to different pathological mechanism and severity. Thus, all pathological mechanisms which plays an important role in process of the diseases are targeted.

On the other hand, since most of these epigenetic alterations are theoretically reversible, the treatment triad in the RTM model will be revealed to be reasonable. Epigenetic processes are the basis of biology and it is believed that they have a high impact on most ADs, and more research about etiology of disorders related scientific study is needed in this area.

Conflict of Interests

The author declares no conflicts of interest in this work.

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