A Pilot Study of Antistress Effects of Vitamin B Complex and Agarwood Extract in an Animal Model with Parallel Observations on Depression in Human Subjects

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Received: February 11, 2021; Accepted: February 25, 2021; Published: March 04, 2021

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Abstract
Background: Depression leads to mood fluctuations and has several causes, such as genetics and chronic stress. Alternative treatments for depression include vitamin B and dietary interventions.
Method: We investigated the effects of vitamin B complex (Tri-B) and dietary agarwood extract (AW) against epinephrine-induced biochemical changes. We analyzed the constituents of AW with gas chromatography–mass spectrometry and performed an AutoDock analysis of the components of AW and Tri-B as potential ligands of the epinephrine receptor. We separately determined the effects of oral AW and intramuscular Tri-B injection on biochemical changes caused by epinephrine in rats. We also observed depression and biochemical changes in human volunteers with or without Tri-B treatment.
Results: The AutoDock outcomes confirmed the improvement in the rats’ biochemical status due to AW and Tri-B binding to the epinephrine receptor. The volunteers treated with Tri-B showed significant amelioration of depression.
Conclusion: Tri-B had a beneficial effect by reducing the levels of tumor necrosis factor-α, interleukin-1 beta, cortisol, cyclooxygenase-2, lipid peroxidation, and nitric oxide in both rats and human volunteers.
Keywords: Depression; Stress; Agarwood; Adrenaline; Cortisol; Vitamin B; TNF-α; IL-1β; COX-2

Introduction
Stress responses of the body to physical or emotional demands may trigger feelings of depression in susceptible people [1,2]. Sustained stress elevates the hormone cortisol and changes the balance between serotonin, dopamine, and other neurotransmitters linked to depression in predisposed people and affects their quality of life [3,4]. Although the etiologies underlying depression are not well understood, a recent review shows that psychological stress can influence this disorder [4]. Depression is a major global public health problem and is frequently comorbid with chronic disorders, such as cancer, stroke, heart conditions, diabetes, and chronic obstructive pulmonary disorder [2]. However, identification and treatment tend to vary worldwide. The role of vitamin B6 as a treatment, both adjuvant and primary, for depression has been considered in several studies [5]. Vitamins, natural diets, and herbs, with an abundance of antioxidant compounds, provide the best alkaline medium to manage chronic and acute inflammation resulting from stress and other conditions [6].

Agarwood (AW) is obtained from the heartwood of Aquilaria (family Thymelaeaceae), a genus of trees native to Southeast Asia, for producing an aromatic resin [6,7]. It is used in medicinal applications to alleviate anxiety, prevent vomiting, treat asthma, and manage digestive, neurodegenerative, and inflammatory conditions [8]. Recent pharmacological investigations have shown that agarwood has various beneficial outcomes, including anti-inflammatory effects [9,10], neuroprotection [11,12], and anti-depressive influences [13,14]. Our recent study showed that AW has anti-inflammation and antiapoptotic effects [6]. The chemical constituents of Cambodian AW chip extract, identified by gas chromatography–mass spectrometry (GC–MS) in our previous study, have advantageous effects. The aromatic compounds of AW include deconexent, hexadecanoic spiro derivative compounds, aristolochene, aromadendrene, octadecanoic acid, and other potential reservoirs of bioactive phytochemical compounds. Most of the compounds identified in AW are antioxidant, anticancer, antimicrobial, and antidiabetic agents [6,7]. Moreover, other chemical compounds of AW, such as agarofuran, α-eudesmol, and guaiol, show antitumor effects, shield against brain damage, and
may be applied in skin lightening preparations. Consequently, AW has the potential to be an effective source of leading compounds to treat stress, anxiety, and depression [7,15,16].

In this study, we investigated the effects of treatment with vitamin B and a dietary AW extract on epinephrine-induced biochemical changes in a rat model and carried out parallel observations of human volunteers administered vitamin B as an intervention for depression. Animal models are extensively used in biochemistry, and the findings of animal studies traditionally form the basis of pilot studies with humans. However, there are concerns about the appropriate range of animals that respond to physiological stimuli in a similar way to humans. From this perspective, our study corresponds to an animal pilot study side-by-side with observations on human responses to vitamin B against depression. The trials aimed to determine the effect of AW and vitamin B on indicators of stress, such as tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), cortisol, cyclooxygenase-2 (COX-2), lipid peroxidation, and nitric oxide (NO), induced by epinephrine in rats. We also observed the differences in the levels of these stress indicators and the liver and lipid profile between human volunteers with vitamin B treatment against depression and those without, and compared the results to those from the rat study.

Materials and Methods

The plant study

The plant materials: We obtained chips of Cambodian agarwood from a local market in Saudi Arabia for this study, supplementary Figure 1. Agarwood chips were crushed and extracted with absolute ethanol according to our previous extraction method using absolute ethanol [6]; we obtained a total AW yield of 39 g.

Gas chromatography mass spectrometry analysis: We followed our previous method for GC–MS analysis to separate the chemical constituents of the AW extract using a gas chromatography system (G3440B, Agilent Technologies, USA) [6]. We then searched the WILEY and NIST (National Institute of Standards and Technology) mass spectral libraries to identify the chemical components of the extract [6]. Furthermore, we screened the unique AW components with AutoDock to determine their potential as ligands of epinephrine receptors to provide more convincing proof of the therapeutic opportunities provided by AW [17,18].

Materials and Methods

Preparation of the modelled epinephrine receptor (PDB: 2rh1), 18 specific AW components, and Tri-B ligands for docking: AutoDock Vina 4.2 software includes procedures for optimizing proteins and ligands [18–21], such as allowing atomic charges to make proteins more polar. Proteins and ligands were adjusted through charge and rotatable bond authorization, consideration of the energy contribution of desolvation through the binding of a ligand to the protein, and previous naming of grid maps of the protein surface for synergy ligands by the auto grid. These tools increase the speed and accuracy of docking with unique scoring capacity, optimize effectively, and perform multithreading of molecular docking [18–21].

The rat study

Chemicals: The following kits were procured: Cortisol ELISA kit (DRG, USA); COX-2 determination kit (catalog kit no.760151, Cayman, USA); Rayo Rat TNF-α ELISA Kit (catalog no. ELR-TNFα-001C, RayBio, USA); and IL-1β kit (Multi Sciences Biotech Co., Ltd., Hangzhou, Zhejiang, China). Tri-B (vitamin B1, B6, and B12 complex) was obtained from a local pharmacy (Merck KGaA, Darmstadt, Germany). N-(1-naphthyl) ethylenediamine, arachidonic acid, sulfanilamide, standard sodium nitrite, sodium dodecyl sulfate, thiobarbituric acid, tetramethoxypropan, epinephrine, and diethylenetriaminepentaacetic acid were purchased from Sigma-Aldrich (USA).

Animals

We used 60 adult male Sprague-Dawley rats weighing 100–110 g in this study. The rats were tested for their health status at 25°C and given a standard diet and water daily for two weeks before the study began. After acclimatization, the rats were divided into six groups of 10 individuals each. All animal experiments were approved by the Experimental Animal Care Society’s Ethics Committee and conformed to the Three Rs (Replacement, Reduction, Refinement) [6,22]. The Institution involved in this work approved the study protocol, animal resources, and experimental procedures. We complied with all institutional and national regulations for the care and handling of laboratory animals [6,22]. The six groups of rats consisted of the following: a control group of untreated rats; an epinephrine group of rats injected with an intramuscular dose of epinephrine at 0.02 g/kg
body mass (bm) once per week for three weeks [5,23,24]; a Tri-B group of rats injected with an intramuscular Tri-B (vitamin B1, B6, and B12 complex) dose at 20 mg/kg bm once per week for three weeks [5,23,24]; an epinephrine+Tri-B group of rats injected intramuscularly with epinephrine and Tri-B at the same dosage and in the same manner as the epinephrine and Tri-B groups for three weeks [5,23,24]; an AW group of rats treated in the same manner as our previous study with a daily oral dose of AW at 100 mg/kg bm for three weeks [6]; and an epinephrine+AW group of rats injected with epinephrine at the same dosage as the epinephrine group plus a daily oral dose of AW as the AW group for three weeks [5,6,23,24].

After the treatment period ended, we withdraw food from the rats for 12 hours before they were anesthetized with diethyl ether and sacrificed. We obtained a liver tissue sample from each rat and rinsed it with a cold saline solution (0.9% NaCl), weighed it, and stored it at −80°C until biochemical examination. We collected an unheparinized blood specimen from each animal and kept it at room temperature for 15 mins, and then serum was sequestered by centrifugation at 3000 rpm at 2°C for 20 min. We stored the serum samples at −30°C until assay.

Biochemical assays

NO level: NO concentration in liver tissue was spectrophotometrically examined according to Montgomery and Dymock [25]; the detailed account of the assay is given in our previous study [6].

Lipid peroxidation: We examined the level of malondialdehyde (MDA), the end product of lipid peroxidation in the liver, using a colorimetric method according to Ohkawa et al. [26]. Our previous study provides the details of this assay [6].

COX-2: We assayed COX-2 enzyme activity in the liver tissue according to Smith et al.’s protocol [27]. The details of this assay are provided in our previous study [6].

LOX: We used a spectrophotometric method for assaying LOX enzyme activity in the liver tissues. For this procedure, we used sodium arachidonate as the substrate according to Haining and Axelrod [28]. The details of this assay are given in our previous study [6].

TNF-α: We evaluated the TNF-α level in the liver tissues using a rat TNF-α ELISA kit according to Brouckaert [29]. See our previous study for further details [6].

IL-1β: According to the manufacturer’s instructions, we measured the IL-1β level in sera using a standard quantitative sandwich ELISA (MultiSciences Biotech Co., Ltd., Hangzhou, Zhejiang, China). We fixed the minimum detectable dose by combining two standard deviations of the mean optical density equivalent to 10 zero standard replicates and estimating the corresponding intensity. We diluted serum samples 1:5 or 1:10 in PBS to obtain pg/ml concentrations. Optical densities were fixed using an absorbance microplate reader (E1x800TM; Bio-Tek Instruments, Winooski, VT, USA) at 450 nm. GraphPad Prism Data Analysis 6 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for data analysis. We examined all materials and described them based on the standard curve [30].

Cortisol level: We estimated the cortisol level in rat serum samples according to the manufacturer’s instructions with an automated chemiluminescence immunoassay system using the Cortisol ELISA kit (DRG, USA). This immunoassay kit allows in vitro quantitative determination of the endogenic cortisol concentration in serum. The system for evaluation of cortisol was a competitive inhibition enzyme immunoassay technique, which has high sensitivity and specificity in the assessment of cortisol levels in rats [31].

Lipid profile: We estimated total cholesterol, low-density lipoproteins (LDL cholesterol [LDL-C]), high-density lipoprotein (HDL cholesterol [HDL-C]), and triglycerides according to Burstein et al., (1970) [32].

Liver function: We spectrophotometrically estimated the aspartate transaminase (AST) and alanine transaminase (ALT) levels in the rat plasma specimens for all the groups using the specific kits described in our previous study [6].

The human study

Patient population and data collection: From 2013 to 2020, this observational study enrolled 238 patients with heart issues, diabetes, migraine headaches, and chronic pain. We assessed the volunteers’ stress, anxiety, and depression status using a questionnaire—the PHQ-9 tool [33]. Patients unable to respond to the questionnaire or with a history of psychiatric diseases or other diseases were excluded from the study. Obese individuals, pregnant women, smokers, and children were also excluded. The selected volunteers were 25–56 years of age, male or female, and had a BMI of 29.98 ± 5.10. The appropriate institutional approved the protocol, which complied with the Helsinki Declaration as revised in 2013. Written informed consent was received from all the volunteers [34]. We divided the volunteers into two groups—Group 1 was not given administered vitamin B complex injections as a treatment (for at least 2–3 months) and Group 2 was administered vitamin B injections (for at least 2–3 months). Each vitamin B ampoule used for therapy by the physician contained as the active components 100 mg of vitamin B1 (thiamine hydrochloride), 100 mg of vitamin B6 (pyridoxine hydrochloride), and 1000 μg of vitamin B12 (cyanocobalamin) in a 3 ml aqueous suspension administered as a single injection per week [5,35]. We collected the data for investigation of parameters in this observational study after each volunteer had received three months of treatment with vitamin B complex.

Measures

Chemicals: The human TNF-α (hTNF-α) enzyme-linked immunosorbent assay (ELISA) test was purchased from Biosource International, Belgium. Human interleukin-1 beta ELISA was purchased from BioVendor (CAT. NO.: RD194559200R). Cortisol EIA kit was purchased from BioSource International, Inc. (Camarillo, USA). The
The human TNF-α (hTNF-α) enzyme-linked immunosorbent assay was used in this study. We estimated the TNF-α levels in the serum with a standard curve created by plotting the absorbance against the concentration of hTNF-α. The significance level of the tests was set at 5% [10,11]. We followed the manufacturer’s instructions for the Human Interleukin-1 Beta ELISA assay (BioVendor) for IL-1β. The absorbance is equivalent to the concentration of IL-1β. A standard curve was created by plotting the absorbance results against the concentrations of IL-1β standards, and the concentrations of unknown samples were calculated from the standard curve [41].

Cortisol level: After we collected fasting blood samples at 8 am, we followed the protocol for measuring the cortisol level with a competitive enzyme-linked immunosorbent assay (ELISA) kit. Cortisol in patient samples or standards were conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody. During substrate incorporation, HRP action generates a blue color whose intensity is equivalent to the amount of cortisol bound to HRP and inversely proportional to the quantity of unconjugated cortisol in the specimens or standards [3].

Lipid profile: We determined the lipid profile of blood samples from the patients after a 12-h fast. We evaluated total cholesterol, triglycerides, HDL-C, and LDL-C with automatic analyzers (cobas c 501 module-Roche Diagnostics) [34].

Liver function: We spectrophotometrically assessed the AST and ALT levels in the human plasma specimens using specific kits as described in Hamouda et al. [34].

Statistical analyses

Data were analyzed using IBM SPSS software version 20.0 (IBM Corp, Armonk, NY). The Kolmogorov–Smirnov test was applied to establish the normality of the distributions of the variables. ANOVA was employed for distinguishing the differences between more than two groups, accompanied by a post hoc Tukey’s test for pairwise comparisons. The significance level of the tests was set at 5% [10,11].

Results

Docking protein (epinephrine receptor PDB: 2rh1) with ligand (phytochemical) molecules

In this study, we found the docking or binding free energy of the epinephrine receptor PDB: 2rh1 (Table 1) and assessed the ligands (Tables 2 and 3), which reflect the binding affinity of the three (Tri-B) and 18 ligands to the receptor PDB: 2rh1 (Table 1). These docking studies showed that among the AW phytochemicals, the stigmastanol component of AW had the highest binding affinity of −11.2 kcal/mole and 2,5-octadecadiynoic acid methyl ester had the lowest at −6.3 kcal/mole. The Tri-B constituents, vitamin B1, vitamin B6, and vitamin B12, had a binding affinity of −7.4, −6.9, and −15.5, respectively (Tables 2 and 4). The binding energy of Tri-B was equal to those of most phytochemical ligands except for vitamin B12, which showed the highest binding energy. Therefore, we chose at least one phytochemical ligand, which confirmed better docking energy, from each separated AW constituent identified by GC–MS. The ligands with the best bioactivity are presented in Table 3.
animal study

Table 5 shows the results for the different parameters studied, given as mean ± SD for 10 rats. Figure 1 shows that epinephrine induced hepatic oxidative stress and inflammation in rats, which was indicated by a significant (p ≤ 0.05) elevation in the levels of NO (174.9%), MDA (572.4%), COX-2 (173.5%), LOX (275.0%), and TNF-α (272.6%), relative to the control group. This was linked to a significant (p ≤ 0.05) elevation in serum total cholesterol (188.6%), triglycerides (100.7%), LDL-C (371.3%), AST (89.5%), ALT (808.3%), and IL-1β (220.4%) compared to the control group, while the epinephrine group showed a significant 24.4% decline in HDL-C compared to the control group (Figures 2a-2c). Consequently, as a side effect of epinephrine injection, oxidative stress and the inflammatory effect led to a 63.2% elevation in serum cortisol levels compared to the control p ≤ 0.05 (Figure 2c).

Figure 2: Biochemical comparison of serum of the different groups in the animal study.

(a) Total cholesterol, triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL); (b) aspartate transaminase (AST) and alanine transaminase (ALT); and (c) cortisol and interleukin-1 beta (IL-1β) levels in six rat groups: the Control group (C) was untreated; the Epinephrine group was injected intramuscularly with epinephrine at a dose of 0.02 g/kg body mass (bm) once per week for three weeks; the Tri-B group was injected intramuscularly with Tri-B (vitamin B1, B6, and B12 complex) at a dose of 20 mg/kg bm once per week; the Epinephrine + Tri-B group was injected intramuscularly with both epinephrine and Tri-B at the same doses and in the same manner as the Epinephrine and Tri-B groups; the AW group was treated orally with a daily dose of agarwood (AW) at 100 mg/kg bm for 21 days; the Epinephrine + AW group was treated with epinephrine at the same dose as the Epinephrine group plus a daily dose of oral AW as the AW group for 21 days. Data are given as the mean ± SD for 10 rats. Statistical significance was set at p ≤ 0.05; means denoted by the same letters are not significantly different.

Administration of Tri-B after epinephrine injection (the epinephrine+Tri-B group) significantly (p ≤ 0.05) reduced the levels of liver NO, MDA, COX-2, LOX, and TNF-α by 40.5%, 31.3%, 39.8%, 63.3%, and 55.7%, respectively, compared to administration of epinephrine only (Figure 1). This was linked to a significant (p ≤ 0.05) drop in serum total cholesterol, triglycerides, LDL-C, AST, ALT, and IL-1β by 53.3%, 43.6%, 28.2%, 39.3%, 81.0%, and 46.4%, respectively, compared to the epinephrine group, while the epinephrine+Tri-B group showed a significant 19.8% elevation in the HDL-C level (Figures 2a-2c). Consequently, the beneficial effect of Tri-B against epinephrine-induced oxidative stress and inflammatory effect led to a 23.1% decline in the serum cortisol level in the epinephrine+Tri-B group compared to that in the epinephrine group p ≤ 0.05; (Figure 2c).

In addition, the administration of AW after epinephrine injection (the epinephrine+AW group) significantly (p ≤ 0.05) reduced the liver NO, MDA, COX-2, LOX, and TNF-α levels by 41.2%, 32.3%, 40.9%, 60.0%, and 55.7%, respectively, compared to treatment only with epinephrine (Figure 1). This was associated with a significant (p ≤ 0.05) decline in serum total cholesterol, triglycerides, LDL-C, AST, ALT, and IL-1β by 44.2%, 42.9%, 29.1%, 31.3%, 31.3%, and 48.0%, respectively, compared to the levels in the epinephrine group (Figures 2a-2c). In comparison, the epinephrine+AW group showed a significant 19.4% elevation in the HDL-C level compared to that of the epinephrine group. In addition, the beneficial effect of AW against epinephrine-induced oxidative stress and inflammatory effect led to a 23.1% decline in the serum cortisol level compared to that in the epinephrine group p ≤ 0.05 (Figure 2c).

In rats injected only with Tri-B for 21 days, the hepatic NO, MDA, COX-2, LOX, and TNF-α levels showed only minor changes by ↑3.8%, ↓10.3%, ↓26.5%, ↑12.5%, and ↓0.8%, respectively, compared to the control group (Figure 1). This was associated with minor changes in serum total cholesterol, triglycerides, LDL-C, AST, ALT, and IL-1β (↑1.6%, ↑2.2%, ↑1.5%, 0.0%, and 0.0%, respectively) compared to the control group, while the Tri-B-only group showed a nonsignificant change of ↑10.6% in the HDL-C level compared to the control (Figures 2a-2c). Furthermore, the Tri-B-only treatment led to a nonsignificant 0.9% decrease in the rat serum cortisol level compared to that of the control group p ≤ 0.05 (Figure 2c).

On the other hand, the rats administered only AW for 21 days (the AW group) showed minor variation in the levels of liver NO (↑1.9%), MDA (↑10.3%), COX-2 (↑26.5%), LOX (↑12.5%), and TNF-α (↑1.5%) compared to the control group (Figure 1). This was associated with minor changes in serum total cholesterol (0.0%), TG (↑3.9%), LDL-C (↑1.1%), AST (↑1.5%), ALT (↑2.8%), and IL-1β (↑10.1%) compared to the levels in the control group. In addition, the AW-only group also showed a nonsignificant change (10.9%) in HDL-C compared to the control group (Figures 2a-2c). Furthermore, administration of AW alone for 21 days led to a minor decrease (↑1.7%) in the rat serum cortisol level compared to that in the control group (Figure 2c). Figure 3 shows the comparison of the therapeutic efficiency of AW and Tri-B in the animal study. The percent of change Epinephrine+Tri-B to Epinephrine in total cholesterol, TG, HDL, LDL, serum cortisol, IL-1β, AST, and ALT showed (↑53.3%, ▼43.6%, ▼19.8%, ▼28.2%,
\[23.1\%, \downarrow 46.4\%, \downarrow 39.3\%, \downarrow 81.0\% \text{ respectively}\) as compared to Epinephrine+AW to Epinephrine percent change \((144.2\%, \downarrow 42.9\%, \downarrow 19.4\%, \downarrow 29.1\%, \downarrow 23.1\%, \downarrow 48.0\%, \downarrow 31.3\%, \downarrow 71.7\% \text{ respectively}\). Also, the percent of change between Epinephrine+Tri-B to Epinephrine in NO, MDA, COX-2, LOX, and TNF-α equal to \((40.5\%, \downarrow 31.3\%, \downarrow 39.8\%, \downarrow 63.3\%, \downarrow 55.7\% \text{ respectively}\) as compared to Epinephrine+AW to Epinephrine showed \((41.2\%, \downarrow 32.3\%, \downarrow 40.9\%, \downarrow 60.0\%, \downarrow 55.7\% \text{ respectively}\), \(p \leq 0.05\) (Figure 3).

3. \(s\%=\% \text{ change from the control group}\)

4. \(#\%=\% \text{ change from the epinephrine group}\)

5. \(F=F \text{ statistic for ANOVA, with post hoc Tukey’s test for pairwise comparison}\)

6. \(*\text{Statistically significant at } p \leq 0.05\)

7. Means indicated by the same letter are not significantly different.

**Abbreviations:** ALT=alanine transaminase; AST=aspartate transaminase; COX-2=cyclooxygenase 2; HDL-C=high-density lipoprotein; IL-1β=interleukin-1 beta; LDL-C=low-density lipoprotein; LOX=lipoxygenases; MDA=malondialdehyde; NO=nitric oxide; TG=triglycerides; TNF-α=tumor necrosis factor.

**The Patient Health Questionnaire (PHQ) results**

The mean (± SD) total score for PHQ-9 was 4.96 ± 5.1 for the Group 1 volunteers compared to 3.54 ± 3.4 for Group 2; a score of 5–9 denoted mild, 10–14 moderate, and ≥15 severe depression; (Group 1 was not given administered vitamin B complex injections as a treatment (for at least 2–3 months) and Group 2 was administered vitamin B injections).

**Biochemical comparison of the two groups in the human study**

Group 2 showed a significant decline in the levels of serum NO (54%), MDA (56.7%), COX-2 (53.6%), LOX (52.9%), IL-1β (51.6%), and TNF-α (30.7%) compared to Group 1 (Figure 4) (Table 6). This was associated with a significant decline (\(p \leq 0.05\)) in serum total cholesterol (7.8%), TG (22.7%), LDL-C (8.3%), AST 8.9%, and ALT (11.7%) compared to Group 1 (Figures 5a and 5b) (Table 6). Group 2 showed a significant 11.6% increase in HDL-C than Group 1 (Figure 5a). Furthermore, the amelioration of oxidative stress and inflammation by Tri-B in Group 2 led to a significant 32% decrease in serum cortisol level compared to Group 1 \(p \leq 0.05\)(Figure 4) (Table 6). Figure 6 compares the therapeutic efficiency of Tri-B between the animal study and the human study. The percent of change Epinephrine+Tri-B to Epinephrine in total cholesterol, TG, HDL, LDL, serum cortisol, IL-1β, AST, and ALT showed \((153.3\%, \downarrow 43.6\%, \downarrow 19.8\%, \downarrow 28.2\%, \downarrow 23.1\%, \downarrow 46.4\%, \downarrow 39.3\%, \downarrow 81.0\% \text{ respectively}\) as compared to group 1 to group 2 \((\downarrow 7.8\%, \downarrow 22.7\%, \downarrow 11.6\%, \downarrow 8.3\%, \downarrow 32\%, \downarrow 51.6\%, \downarrow 8.9\%, \downarrow 11.7\% \text{ respectively}\). Also, the percent of change between Epinephrine+Tri-B to Epinephrine in NO, MDA, COX-2, LOX, and TNF-α equal to \((40.5\%, \downarrow 31.3\%, \downarrow 39.8\%, \downarrow 63.3\%, \downarrow 55.7\% \text{ respectively}\) as compared to the percent of change between (Epinephrine+Tri-B) and Epinephrine compare with the percent of change between (Epinephrine+AW) and Epinephrine. rats: the Epinephrine group was injected intramuscularly with epinephrine at a dose of 0.02 g/kg body mass (bm) once per week for three weeks; the Tri-B group was injected intramuscularly with Tri-B (vitamin B1, B6, and B12 complex) at a dose of 20 mg/kg bm once per week; the Epinephrine + Tri-B group was injected intramuscularly with both epinephrine and Tri-B at the same doses and in the same manner as the Epinephrine and Tri-B groups; AW=rats treated orally with a daily dosage of agarwood (AW) at 100 mg/kg bm for 21 days; Epinephrine+AW=rats treated with epinephrine at the same dosage as the epinephrine group plus a daily dose of oral AW as the AW group for 21 days.

**Notes**

1. Data are given as mean ± SD for 10 rats.

2. Control=untreated rats; Epinephrine=rats injected with an intramuscular dose of epinephrine at 0.02 g/kg body mass (bm) once per week for three weeks; Tri-B=rats injected with an intramuscular Tri-B (vitamin B1, B6, and B12 complex) dose at 20 mg/kg bm once per week; Epinephrine+Tri-B=rats injected intramuscularly with a dose of both epinephrine and Tri-B using the same dosage and in the same manner as the epinephrine and Tri-B groups; AW=rats treated orally with a daily dose of agarwood (AW) at 100 mg/kg bm for 21 days; Epinephrine+AW=rats treated with epinephrine at the same dosage as the epinephrine group plus a daily dose of oral AW as the AW group for 21 days.

**Figure 3:** Comparison of the therapeutic efficiency of agarwood (AW) and Tri-B (vitamin B1, B6, and B12 complex) in the animal study.

Total cholesterol, triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate transaminase (AST), alanine transaminase (ALT), cortisol, interleukin-1 beta (IL-1β), nitric oxide (NO), malondialdehyde (MDA), cyclooxygenase 2 (COX-2), lipoxygenases (LOX), and tumor necrosis factor (TNF-α) levels in the percent of change between (Epinephrine + Tri-B) and Epinephrine compare with the percent of change between (Epinephrine + AW) and Epinephrine. rats: the Epinephrine group was injected intramuscularly with epinephrine at a dose of 0.02 g/kg body mass (bm) once per week for three weeks; the Tri-B group was injected intramuscularly with Tri-B (vitamin B1, B6, and B12 complex) at a dose of 20 mg/kg bm once per week; the Epinephrine + Tri-B group was injected intramuscularly with both epinephrine and Tri-B at the same doses and in the same manner as the Epinephrine and Tri-B groups; the AW group was treated orally with a daily dose of agarwood (AW) at 100 mg/kg bm for 21 days; the Epinephrine + AW group was treated with epinephrine at the same dose as the Epinephrine group plus a daily dose of oral AW as the AW group for 21 days. Data are shown as mean ± SD for 10 rats. Statistical significance was set at \(p \leq 0.05\); means denoted with the same letters are not significantly different.

**Figure 4:** Comparison of the therapeutic efficiency of Tri-B between the animal study and the human study. The mean (± SD) total score for PHQ-9 was 4.96 ± 5.1 for Group 1 volunteers compared to 3.54 ± 3.4 for Group 2; a score of 5–9 denoted mild, 10–14 moderate, and ≥15 severe depression; (Group 1 was not given administered vitamin B complex injections as a treatment (for at least 2–3 months) and Group 2 was administered vitamin B injections).
group 1 to group 2 showed (↓54%, ↓56.7%, ↓53.6%, ↓52.9%, ↓30.7% respectively), p ≤ 0.05 (Table 6) (Figure 6).

Figure 4: Biochemical comparison of the two groups in the human study.

Cortisol, interleukin-1 beta (IL-1β), nitric oxide (NO), malondialdehyde (MDA), cyclooxygenase2 (COX-2), lipoxygenases (LOX), and tumor necrosis factor (TNF-α) levels were compared between Group 1 (without vitamin B complex treatment) and Group 2 (with vitamin B complex treatment). Data are shown as mean ± SD for 119 volunteers in each group. Statistical significance was set at p ≤ 0.05.

Figure 5: Comparison of lipid and liver profiles between the two groups in the human study.

(a) Total cholesterol, triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) and (b) aspartate transaminase (AST) and alanine transaminase (ALT) levels in Group 1 (did not receive vitamin B complex treatment) and Group 2 (with vitamin B complex treatment). Data are shown as mean ± SD for 119 volunteers in each group. Statistical significance was set at p ≤ 0.05.

Notes
1. Data are shown as mean ± SD
2. Group 1=without vitamin B complex treatment; Group 2=with vitamin B complex treatment
3. t=Student’s t-test
4. *Statistically significant at p ≤ 0.05

Abbreviations: ALT=alanine transaminase; AST=aspartate transaminase; COX-2=cyclooxygenase2; HDL-C=high-density lipoprotein; IL-1β=interleukin-1 beta; LDL-C=low-density lipoprotein; LOX=lipoxygenases; MDA=malondialdehyde; NO=nitric oxide; TG=triglycerides; TNF-α=tumor necrosis factor.

Discussion
Stressful life circumstances have a role in creating and developing depression as a result of multiple synergies between environmental stressors and individual vulnerability factors. Gustavo and Charles (2016) have shown the role of these factors and their potential synergies at the interface between chronic stress and depression. Genetic factors, including different single-nucleotide polymorphisms (SNPs), may be associated with functional and structural modifications in specific neural structures, including enhanced amygdala reactivity and reduced hippocampus function [1]. Environmental factors affect individuals in various ways, triggering an adaptive response to stress, which depends on the psychological and biological character of the interaction between stressors and individual resources. Psychological processing includes all cognitive processes associated with incoming learning and previous experiences, the subjective evaluation of different characteristics related to stressors, such as their chronicity, predictability, and controllability, and the potential means to cope with them. Biological mediation includes activation of various neural structures underlying information processing, including sensory pathways, which carry environmental input to the central nervous system (CNS), and the resulting activation of neural and neuroendocrine cascades of molecular events, mediated by the consequent activation of the hypothalamic–pituitary–adrenal (HPA) axis [1,42]. An extreme and prolonged chronic stressful situation may lead to hyperactivity of HAX, decreases in monoamine oxidase, and increases in proinflammatory cytokines; the finding in this study for the group with epinephrine-induced stress, which showed elevated oxidative stress and inflammatory markers compared to the control group, agrees with this. This situation may lead to maladaptive settings, which may increase pathological conditions such as inflammatory disorders, autoimmune disorders, anxiety, and mood disturbances, including depression, especially in individuals with increased genetic vulnerability and other conditions [1,43]. Inflammatory processes are also associated with adaptive responses to stressful circumstances, with resulting synthesis and discharge of proinflammatory cytokines. This leads to excess cortisol secretion, which modulates and prolongs the stress response, causing more maladaptive alterations of the neural and neuroendocrine systems and the development of depression symptoms, especially in chronically stressed individuals; the findings in our study agrees with this. Our results shown in Figure 1 concur with previous reports that acute and chronic psychosocial stress may activate inflammatory responses and the proinflammatory cytokines interleukin-1, interleukin-6, and tumor necrosis factor-alpha [1, 44]. We found higher levels of MDA, NO, TNF, COX-2, LOX, and IL-1β in the epinephrine group than in the control group in the rat study and in Group 1 volunteers than in Group 2 volunteers (Figure 4). A previous study reported that acute stress affects the endocrine, immune, and metabolic functions in humans, and that mood plays a causal role in the modulation of responses to...
acute stress. Our findings are in accordance with this and showed an increase in metabolic functions of the liver and the lipid profile as well as cortisol in the epinephrine group compared to the control group in the rat study and in Group 1 volunteers compared to Group 2 (Figures 2, 4a, 4b, and 5) [45].

The role of proinflammatory cytokines in chronic stress, which leads to the development of depression, has been explained by various investigations in relation to their potential mechanisms of action. Environmental stressors activate the sympathetic part of the autonomic nervous system, with the resulting release of catecholamines, such as epinephrine that stimulates receptors on immune cells and proinflammatory cytokines. Chronic inflammatory responses in the CNS may result in the excessive release of proinflammatory cytokines, leading to reduced neurotrophin concentrations and neuroplasticity [46,47]. To initiate stress that can trigger feelings of depression, we injected the rats in this study with epinephrine once a week for three weeks; after the confirmed potential relationship between epinephrine and the treatment compounds, we used AutoDock analysis to resolve the induced depression. We first attempted AutoDock analysis in the animal study to conform to ethical standards of Replacement, Reduction, and Refinement in scientific tests.

Molecular docking is a theoretical simulation system based on bioinformatics, which considers the interaction between molecules (such as ligands and receptors), and predicts their binding modes and affinity through computer analysis. Molecular docking serves as an acceptable mechanism in medicinal chemistry for areas such as structure-based logical drug design. Numerous primary investigations correlating biomolecular interactions in the food matrix and phytochemistry have gradually developed during recent years. The extraordinary advantages of molecular docking, such as prediction of experiments with fewer numbers of trials and prevention of loss of material in trials, have attracted increasing attention to its potential application in various areas. In docking complex binding energy, the lowest energy positions indicate the highest binding affinity, as high energy produces unstable conformations that help in pharmacological trials [48–51].

Pharmacological treatment of depression, mostly with selective serotonin reuptake inhibitors, with or without psychotherapy, has become possible over the past 20 years. Complementary and alternative medicine includes nutraceuticals, meditation, massage, homeopathy, and other procedures beyond the field of conventional clinical care. Among these interventions for depression are dietary interventions and vitamin supplementation, particularly with B vitamins. Deficiencies in thiamine, folate, B12, and B6 are indicated in psychiatric symptoms and their proper supplementation is recognized. Several studies have examined the correlation between folate levels and psychiatric disorders, especially in therapy for depression [52].

Vitamin B6, including pyridoxal, pyridoxamine, and pyridoxine, is an alternative treatment, which may be a significant factor in hormone-related depression through its role in the proper metabolism of particular neurotransmitters thought to be involved in depression. Deficiencies in B6 have been shown to occur in women with hormone-related depression (i.e., premenstrual syndrome and premenstrual dysphoric disorder), and are connected to indications of discomfort. Based on the relationship between deficiency in B6 and common symptoms of depression and its function in the metabolism of carbohydrates and gonadal steroids, it has been hypothesized that B6 may be successfully used in the management of hormone-related depression [53,54]. On the other hand, studies have reported that folic acid and vitamin B12 supplementation may be associated with an increased risk of colorectal cancer [53–55].

Other investigations have reported that Tri-B (B1, B6, and B12) injection following epinephrine improved malondialdehyde and blood sugar levels in stressed rats [5]. The results of this study agree with this finding. Lipid peroxidation, NO, TNF-α, IL-1β, cortisol, COX-2, LOX, AST, ALT, and lipids declined in rats after separate treatment with Tri-B and AW extract compared to treatment with only epinephrine. Simultaneously, compared to the control group, the change in the AW and Tri-B groups was minor, which may be due to the dose and period of injection in healthy rats in vivo. This result for the animal study is confirmed by the results for the human study; there was a significant improvement in symptoms stated in PHQ-9, the depression questionnaire, as well as in serum parameters in volunteers treated with Tri-B compared those that did not receive the treatment and the reference ranges (Figures 4 and 5).

As vital regulators of brain–body interaction, immune mediators such as the cytokines IL-1β, IL-6, soluble IL-2 receptors, and TNF-α, which affect diverse CNS functions required in cognition, sleep, and the brain network reward, are elevated in depressive patients. TNF-α can stimulate the HPA axis, directly elevate indoleamine-2,3-dioxygenase expressed in macrophages and dendritic cells in the brain, and catabolize tryptophan, which is the substrate for serotonin synthesis, through the kynurenine pathway. Previous studies identified that, TNF-α and IL-1β promote imbalance, serotonin uptake in the mouse midbrain, and striatal synaptosomes by activating serotonin transporters [56–58].

Stress is an induced imbalance of neurotransmitters, hormones, and cytokines that intensifies autoimmune responses [59–61]. Previous reports have suggested that acute and chronic stress raises proinflammatory cytokine storm signaling. The results of this study agree with the signaling of cytokines, such as IL-1β, IL-6, and TNF-α as well as cascade signaling of COX2, LOX, and free radicals in rheumatoid arthritis and multiple sclerosis, and the fueling of cytokine storms by the stress hormone cortisol and adrenaline neurotransmitters [62–64]. The outcomes of cytokine storms can be fever, low blood pressure, damage to the heart, and, in some circumstances, organ failure and death [62,63]. Accordingly, there is high interest in understanding the underlying mechanisms to find ways of blocking cytokine storms without altering the effectiveness of therapies.
for disorders. Previous studies suggest that dietary intake of vitamins, especially B vitamins including B6, B9, and B12, may significantly affect mood and stress [5,63,65]; our study also found that there are significant ameliorating effects of AW and Tri-B against the effects of epinephrine in the rat study and significant improvement of depression symptoms in human volunteers treated with Tri-B. Phyto-constituents can control and modify numerous biological activities by interacting with molecules required in signaling pathways [6,62]. This study demonstrated the potential efficacy of vitamin B complex and AW phytochemicals (the stigmasterol AW compositions showed the highest binding energy) as a treatment for stress and depression; first, molecular docking of selected phytoconstituents with the epinephrine receptor identified potential ligand molecules, and second, the study found that these compounds alter the effect of epinephrine in rats. Tri-B had a beneficial effect against epinephrine-induced stress more than AW extract that confirmed by AutoDock analysis, where the Mean binding energy of affinity mod of Tri-B synergetic effect more than AW extract synergetic effect (Table 4) (Figure 3). The results of the animal study were confirmed by the finding of the human study; there was a significant improvement in serum parameters of volunteers treated with Tri-B compared to those of the volunteers without the treatment and reference ranges (Figure 6).

![Figure 6: Comparison of the therapeutic efficiency of Tri-B between the animal and human studies.](image)

Total cholesterol, triglycerides (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate transaminase (AST), alanine transaminase (ALT), cortisol, interleukin-1 beta (IL-1β), nitric oxide (NO), malondialdehyde (MDA), cyclooxygenase2 (COX-2), lipoxygenase (LOX), and tumor necrosis factor (TNF-α) levels were compared between the percent of change between (Epinephrine + Tri-B) and Epinephrine in rat study compare with the percent of change between (group 1) to (group 2) in human study. The Epinephrine group was injected intramuscularly with epinephrine at a dose of 0.02 g/kg body mass (bm) once per week for three weeks; the Tri-B group was injected intramuscularly with Tri-B (vitamin B1, B6, and B12 complex) at a dose of 20 mg/kg bm once per week; the Epinephrine + Tri-B group was injected intramuscularly with both epinephrine and Tri-B at the same doses and in the same manner as the Epinephrine and Tri-B groups.

Group 1 (without vitamin B complex treatment) and Group 2 (with vitamin B complex treatment). Data are shown as mean ± SD for 119 volunteers in each group. Statistical significance was set at p ≤ 0.05.

This study measured the effect of epinephrine after 21 days to assess chronic stress that may lead to depression. We assessed the potential binding of ligands to the β2 adrenoreceptor (2rh1) by AutoDock analysis. It has been reported that epinephrine (adrenaline) reacts with α- and β-adrenoceptors, prompting vasoconstriction and vasodilation in both cases, with greater sensitivity to β receptors. Epinephrine secretion in the body is at a lower level than pharmacological doses. Although α receptors are less sensitive to epinephrine at pharmacological treatment doses, many peripheral α1 receptors lead to higher circulating epinephrine levels than β-adrenoceptors. Epinephrine and norepinephrine are receptor ligands to α1-, α2-, or β-adrenoreceptors. α1 couples with Gq, a guanine nucleotide-binding protein, which acts as a molecular switch inside cells. Gq is involved in relaying signals from various stimuli from the outside to the inside of cells, which results in elevated intracellular Ca2+ and subsequent smooth muscle contraction [1,43,66].

Moreover, α2, which couples to Gi, creates a reduction in neurotransmitter discharge and a decrease in the second messenger cAMP activity following smooth muscle contraction. On the other hand, β receptors bind to Gs and improve intracellular movement of cAMP, which ends in smooth muscle relaxation, heart muscle contraction, and glycogenolysis, the release of glycogen from glucose for energy; they also increase lipolysis. Generally, the binding of adrenergic receptors stimulates the sympathetic nervous system (SNS) in the fight-or-flight response. However, the constant chronic stimulation of the SNS has side effects and increases proinflammatory cytokines [1,43].

In pharmacology, an agonist ligand binds to and triggers a physiological response; on the other hand, an antagonist ligand binds to a receptor but does not stimulate a physiological reaction. In contrast, an inverse agonist is a ligand that binds to the receptor, inhibits its usual activity, and causes an effect opposite to that of the agonist. Binding affinity, potency, and efficacy can decide the overall strength of a drug. The influence of a ligand results from the complex interaction between binding affinity and ligand efficacy. It elicits a biological response upon adhering to the target receptor. The quantitative significance of this response may be agonist, antagonist, or inverse agonist, and a physiological response is accordingly generated. Selective ligands bind to limited types of receptors; in contrast, non-selective ligands bind to numerous varieties of receptors and tend to have multiple adverse influences because they bind to many other receptors as well as to the one causing the desired effect [67]. The rat and human investigations in our study showed that the controlling effect of vitamin B and AW extracts may be due to their role as inverse agonists re-
versing the induction of stress and depression by epinephrine; there was a drop in lipid peroxidation, NO, TNF-α, IL-1β, cortisol, COX-2, LOX, AST, ALT, and lipids in the rats separately administered Tri-B and AW extract compared to the epinephrine group. Simultaneously, there was a nonsignificant change in health in vivo in the AW and Tri-B groups compared to the control group. According to our previous work, AW contains essential oils, including cyclohexanone derivatives, with many medicinal properties, such as sedative, anti-inflammatory, and antiapoptotic effects [6].

With the aid of docking analysis, we report compounds with potential binding affinity with the beta receptors of epinephrine; this result was confirmed by biochemical analyses, which showed a synergetic inhibitory effect of AW compounds against epinephrine in the rat study. Agarwood’s amelioration of the effects of epinephrine suggests that its extract may act as an inverse agonist. Doconexent is used as a supplement containing high levels of docosahexaenoic acid (DHA), which has anti-inflammatory effects and plays a vital role in the development and function of the cerebral cortex, skin, and retina [68]. The ester form of hexadecanoic acid, also known as palmitic acid, is used to treat schizophrenia [69]. Oleic acid, (Z,Z)-9,12-octadecadienoic acid, hexadecenoic methyl ester, quinazolin-4-, and aromadendrene oxide derivatives have antibacterial and antifungal properties and other beneficial effects on tumors and autoimmune and inflammatory conditions [70–72]. Agarwood also contains stigmasterol, a phytosterol, which shows the highest potential binding energy with epinephrine receptors. Many previous studies have shown that it reduces the risk of cardiovascular diseases and LDL cholesterol and has a role as an antiangiogenic and antitumor agent by downregulating TNF-α signaling [73,74].

The relationship between the responses of inflammatory cytokines and cortisol is a bidirectional feedback loop involving end products, cytokines, and cortisol. For instance, exposure to a stressor leads to the release of specific inflammatory cytokines, such as IL-10, TNF-α, and IL-6, and induces the HPA axis and cortisol discharge. Furthermore, cortisol’s anti-inflammatory effects then feed back and suppress the further release of cytokines [75]; this effect was shown in our study where a decline in the serum cortisol level was associated with a decline in inflammatory parameters after treatment with vitamin B complex in both rats and humans, as well as after oral administration of AW to rats following stress induction. Furthermore, sustained stress leads to inflammation and high physiological levels of cortisol, with resulting fluctuations in the anti-inflammatory effect of cortisol and decreased tissue sensitivity to it. As a consequence, stressed individual immune cells are less sensitive to cortisol. In addition, the body’s regulation of the inflammatory response of the immune system is inadequate [76]. Previous work reported that sustained inflammation, stress, or depression leads to extremely high production of cytokines, such as IL-6 and TNF-α, and a drop or change in their response to cortisol therapy in patients with rheumatoid arthritis and other disorders [64,75,76]. The previous study agrees with current work where there is an increase in inflammatory parameters, including TNF-α, IL-1β, cortisol, COX-2, lipid peroxidation, NO after stress-induced in rats as well in volunteers subjects separately study. Furthermore, there is an improvement in the animal’s biological tests due to AW and Tri-B against epinephrine receptors, and significantly ameliorating volunteer treatment with Tri-B was observed. Tri-B’s parallel beneficial on the level of IL-1β, IL-6, TNF-α, and cortisol, and cascade signaling of COX2, LOX in volunteers, compared with rats results, this may be due to the combination of agarwood phytochemical as well Tri B.

Conclusion

We confirmed control of epinephrine-induced oxidative stress and inflammation by vitamin B and the AW extract by the docking of binding free energy of the epinephrine receptor, 2rh1, with the ligands of vitamin B and the AW extract. Furthermore, there was a decline in lipid peroxidation, NO, TNF-α, IL-1β, cortisol, COX-2, LOX, AST, ALT, and lipids in rats after separate treatment with Tri-B and AW extract compared to the rats treated with epinephrine. The results of the animal study were confirmed by comparisons of data for human volunteers treated with Tri-B with those for untreated volunteers and reference ranges. We conclude that Tri-B and AW extract are primary point treatments for the prevention/management of stress, depression, and neurodegenerative conditions.

Acknowledgments

AFH is grateful to Taymour-Lank M. Farawilla for help with the AutoDock analysis, and solving technical problems during the process. AFH acknowledges the support of the Poison Control and Medical Forensic Chemistry Center, Ministry of Health, Jazan, Saudi Arabia for the chromatography part of this study. AFH thanks Amgad Hamza for help with statistical analyses and is deeply grateful to all the doctors who helped in this research and for the willingness of the volunteers to take part in this study.

Author Disclosure Statement

The author declares that there are no competing financial interests.

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