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INTRODUCTION

Viburnum genus belonging to the Caprifoliaceae family, consist of about 230 species all across the Southeast Asia and South America [1]. It constitutes four different species in the native of Turkey; which includes Viburnum lantana L., Viburnum orientale Pallas, Viburnum tinus L., and Viburnum opulus L. [2]. The genus has medicinal uses ranging from antispasmodic, diuretic, hepatoprotective, anti-inflammatory, and antiseedative effects [3,4]. A local drink called “gilaburu” was formulated from the fruit of V. opulus in Middle Anatolia while the bark of V. lantana is known for its analgesic potential [5]. The antioxidative property of V. dilatatum was observed in rats exposed to stress [6] and in diabetic rats [7]. The genus Viburnum has been reported to contain a vast array of phytochemicals such as diterpenoids [8,9], triterpenoids [10,11], iridoids [12], sesquiterpenes [13], and polyphenols [1]. The various biochemical activities of the plant genus are probably associated with the phytochemicals it contains. Out of all the secondary metabolites reported to be contained in the genus Viburnum, the polyphenols have gained much awareness in recent years owing to the fact that they display a vast array of biological activities which includes antibacterial, antioxidant, antimicrobial, anti-inflammatory, and anti-thrombotic properties [14]. This study is on V. opulus (L) which is also known as snowball tree or the guilder rose [15]. The antioxidant activity of V. opulus has been reported [16]. This study will be the first where in the bioactive compounds of V. opulus are ascertained via gas chromatography-mass spectrometry (GC-MS) technique; moreover, no literature has also documented its toxicity evaluation in albino Wistar rats. It is, therefore, imperative to access the sub-acute toxicity of the leaf extract of V. opulus in rats and furthermore determine its phytochemical and antimicrobial profiles in different solvent fractions.

METHODS

Plant materials
Fresh green leaves of V. opulus (Egungun eja) were obtained from Benja Community, Ado-Odo Ota, Local Government Area, Ogun State, Nigeria. The plant was authenticated by a taxonomist, Dr. A. C. Omonhinmin in Biological Sciences Department, Covenant University.

Extract preparation
The fresh leaves were allowed to dry for 2 weeks under room temperature and subsequently pulverized to powder form. 666 g of the pulverized leaves were soaked in 90% ethanol for 72 hrs, and a 10% yield of the ethanolic extract was obtained at reduced pressure of 50°C with the aid of a rotary evaporator. The crude ethanolic extract was thereafter subjected to sequential fractionation using hexane, ethyl acetate, and butanol, respectively, to obtain hexane, ethyl acetate, butanol, and water fractions [17]. The different fractions were used for the GC-MS analysis.

Phytochemical analysis

Different fractions of V. opulus leaves were exposed to phytochemical analysis to detect the existence of any of the following: saponins, alkaloids, proteins, carbohydrates, coumarin, amino acids, anthraquinone glycosides, tannin, flavonoids, phenolic compounds terpenes, quinone, and using standard procedures [18].

GC-MS analysis

This was performed using a GC (Hewlett Packard; model 6890 series) having an ionization flame detector, injector with a temperature of 250°C. Temperature was set at 50°C for 5 minutes and further increased at a steady rate of 2°C/minutes. The gas used was 99.9% helium gas after which 1 µm of the extract was injected at a ratio of 1 to 30 (1:30).
The GC (Model 6890 series) was equipped with NIST14.L library software database.

Antimicrobial activity
Antimicrobial activity of the different fractions was performed using agar well diffusion method and this was tested against Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Bacillus spp., and Micrococcus spp. Bacterial culture (16 hrs old) was swabbed on the prepared Muller-Hinton agar (Hi Media, India) plates and left to dry for about 5 minutes. Using a sterile cork borer, 4 wells 5 mm in diameter were drilled on the plates. A total concentration of 30 mg/ml of extract was prepared by dissolving in their appropriate solvents. Varying concentrations of the extract (300, 600, 900, and 1200 µg) were stacked onto the wells, allowing it to circulate for about 10 minutes. These were further incubated at 37 °C for 24 hrs. The width of inhibition zone was estimated and recorded after incubation.

Experimental animals
A total of 35 healthy male albino Wistar rats with weight ranging from 82 to 190 g, were obtained at the Animal breeding centre, Federal University of Technology, Abeokuta, Ogun State, Nigeria. The rats were kept in a well-aerated room with standard conditions (12/12 hrs light/dark cycle, 25°C and 65% humidity), and fed with quality rat chow including water (Graceline feeds, Ota, Ogun state). The animals were left to adjust to the environment for 2 weeks during which they were assessed for morphological/behavioral changes before the inception of the experiment. The animals were handled following the recommendations for the use and care of laboratory animals stated by the National Institutes of Health [19].

Sub-chronic toxicity studies
The method according to Adebayo et al. [20] was adopted. Thirty-five (35) male albino Wistar rats were divided into five groups containing seven rats per group. The control (Group A) was administered 1 ml of distilled water. Groups B to E orally received varying concentrations of the extract for 28 days. At the close of the administration, food was withdrawn from the rats for 12 hours after the last dose (250, 500, 1000, and 1500 mg/kg body weight) of the extract for 10 minutes. These were further incubated at 37 °C for 24 hrs. The width of inhibition zone was estimated and recorded after incubation.

GC-MS analysis of ethanol, butanol, hexane, and ethyl acetate fractions
GC-MS analysis of ethanol, butanol, hexane, and ethyl acetate fractions were shown in Fig. 1a-d. This revealed different peaks. GC-MS analysis of the butanol fraction revealed 34 bioactive compounds (Fig. 1c). First compound identified, with less retention time (RT) (7.40 minutes) in the butanol fraction, was diethyl phthalate having 26 different but very close RTs. The predominant compound was diethyl phthalate having 26 different but very close RTs.

RESULTS
Result obtained from the phytochemical analysis is as shown in Table 1. The hexane fraction showed the existence of saponins, glycosides, steroids, tannins, quinines, and phenols while the ethyl acetate fraction showed the presence of alkaloid, phenol, coumarin, and steroids. The butanol fraction revealed the presence of tannin, saponin, alkaloid, terpenoids, and phenols.

The ethanolic extract, butanol, and water fraction of V. opulus showed some level of inhibitory activity against most of the microorganism tested (Table 2). The organisms were, however, resistant to the N-hexane and ethyl acetate fraction. The diameter of the zone of inhibition varied from 4 to 9 mm.

Statistical analysis
Data were expressed as mean±standard error of mean and analyzed with one-way ANOVA along with Tukey’s post-hoc using the Statistical Package for Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA). p<0.05 was considered statistically significant.

Table 1: Phytochemical analysis of fractions of V. opulus leaves

| S.No. | Phytochemicals | Ethanol extract | N-hexane extract | Ethyl acetate extract | Butanol extract |
|-------|----------------|-----------------|------------------|----------------------|----------------|
| 1     | Carbohydrate   | -               | +                | -                    | -              |
| 2     | Tannin         | +               | +                | -                    | +              |
| 3     | Saponin        | -               | +                | +                    | -              |
| 4     | Alkaloid       | -               | +                | +                    | +              |
| 5     | Flavonoids     | +               | -                | -                    | -              |
| 6     | Quinones       | -               | +                | -                    | -              |
| 7     | Glycoside      | -               | +                | -                    | -              |
| 8     | Cardiac glycoside | -       | -                | -                    | -              |
| 9     | Terpenoids     | +               | -                | -                    | +              |
| 10    | Phenol         | -               | +                | +                    | -              |
| 11    | Coumarin       | +               | -                | +                    | -              |
| 12    | Steroids       | +               | +                | +                    | -              |

*: Positive, -: Negative, V. opulus: Viburnum opulus

Table 2: Zone of inhibition of fractions of V. opulus against some bacteria

| Organism                  | EF (mm) | NF (mm) | EAF (mm) | BF (mm) | WF (mm) |
|---------------------------|---------|---------|----------|---------|---------|
| Escherichia coli          | 8       | -       | -        | 7       | 6       |
| Staphylococcus aureus     | 7       | -       | -        | 9       | -       |
| Proteus vulgaris          | 8       | -       | -        | 6       | 5       |
| Pseudomonas aeruginosa    | 6       | -       | -        | 6       | 4       |
| Bacillus spp.             | 8       | -       | -        | -       | 4       |
| Micrococcus spp.          | 4       | -       | -        | 6       | 3       |

EF: Ethanollic fraction, NF: N-hexane fraction, EAP: Ethyl acetate fraction, BF: Butanol fraction, WF: Water fraction, V. opulus: Viburnum opulus

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Biochemical parameters determination
Biochemical parameters such as alkaline phosphatase [21], aspartate aminotransferase (AST) [22], alanine aminotransferase (ALT) [23], total bilirubin [24], albumin [25], urea [26], low-density lipoprotein (LDL) [27], high-density lipoprotein (HDL), and cholesterol [28] were assayed by using an auto-analyzer (Archem BM240, Turkey).

Statistical analysis
Data were expressed as mean±standard error of mean and analyzed with one-way ANOVA along with Tukey’s post-hoc using the Statistical Package for Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA). p<0.05 was considered statistically significant.
other prevalent compounds present were pentadecane 2,6,10,14 tetramethyl (6.22%), squalene (13.15% and 5.47%) (Fig. 1b).

The hexane fraction revealed 27 compounds; the first compound with the least RT (6.16 minutes) was heptane, 2,6-dimethyl-oxalic acid, and isobutyl nonyl ester (2.12%) while the prominent one was phytol (15.37%) having RT of 23.84 minutes.

Finally, from the ethanol crude extract, 14 different compounds were identified via the GC-MS. The major one was phytol (58.55%) which was also present in other fractions (Fig. 1a). Table 3 shows the RT along with the structures of the phytochemical constituents identified by GC-MS spectra in the ethanolic extract of *V. opulus*.

**Biochemical analysis**

The result of the toxicity study showed no significant elevation in ALT and AST level (p<0.05) (Fig. 2a and b) in all treated groups. The ethanolic extract of *V. opulus* significantly increased the HDL level in the serum (p<0.05) (Fig. 3b) while no significant change in the serum LDL level was observed (Fig. 3a). There was a reduction in the serum triglyceride level but not significant (Fig. 3c). Furthermore, there was a dose-dependent significant increase in serum albumin levels in the 500, 1000, and 1500 mg/kg groups (Fig. 2d) while the reduction observed in the urea levels in all treated groups was not significant (Fig. 2e).

**Histopathological studies**

No cellular nor fibrotic damage of rats’ liver tissues was detected across all treatment groups in comparison with the control group (Fig. 4a-e).

**DISCUSSION**

*V. opulus* L. is cultivated as an ornamental plant in different countries, and the dried fruits have been reported to contain organic acids and phenolic glucoside which were used to treat uterine cramps and used as general pain killer [29]. The biological activities of medicinal plants are associated with the type and nature of secondary plant metabolites they contain. The phytochemical investigation which showed the presence of tannins, saponins, phenols, flavonoids, terpenoids, and steroids (Table 1) is in line with the previous studies as reported by Yilmaz et al. [4]. From the antimicrobial screening (Table 2), the butanol fraction was the most effective against the entire microorganism tested having a zone of inhibition ranging from 6 to 9 mm as compared with other fraction with no activity. The presence of saponin might be responsible for it antimicrobial effect as saponin has been reported to have an inhibitory effect on Gram-positive bacteria [30]. It is interesting to note that the butanol fraction was active against *S. aureus*, an infectious agent associated with several animal and human infections. Furthermore, the saponins from the ethanolic crude extract are complementary to standard antibiotics like the penicillin used in this study.

The GC-MS analyses, as shown in Fig. 1c of the butanol fraction identified some phytochemical compounds such as octadecane (0.19%), hexadecane (0.46%), benecosane (0.24%), 9-octadecenoic acid(Z)-, methyl (0.41%), phytol (1.15%), phthalic acid, monooctyl ester (0.49%), and 1H-Indole, 5-methyl-2-phenyl- (0.60%) with the longest RT of 30.34. Phytol which is one of the compounds identified in the butanol fraction; is an essential component of plants that is used in making toilet soaps, shampoo, and general household cleaners as a result of its high antimicrobial against food borne pathogens [31] and also a precursor in manufacturing synthetic forms of vitamin K and vitamin E [32,33]. Inoue et al. [34] reported phytol to have antibacterial activity against *S. aureus* by causing cell membrane damage; this is in line with this study where the butanol fraction was found to be active against *S. aureus*. The water fraction contains bioactive such as oxalic acid, allyl tridecyl ester (1.27%), heptane, 2,6-dimethyl- dodecane (2.59%), octane, 3,6-dimethyl-(1.81%), tridecane (2.79%), decahydro-1,1a,5,6-pentamethylnaphthalene (1.39%), dodecane, 2,6,10-trimethyl- (1.6%), tetradecane (3.34%), and 2-pentane, 4-cyclohexylidene-3,3 -diethyl- (0.67%). The hexane fraction analyzed by GC-MS identified compounds such as tridecane (2.77%), dodecane, 2,6,10-trimethyl (1.55%), tetradecane (2.73%), 2,6,10-trimethyledecane (2.89%), pentadecane (2.92%),
hexadecane (2.27%), tridecane, 5-propyl (1.6%), heptadecane (2.64%), pentadecane, 2,6,10,14-tetramethyl (5.54%), octadecane (2.64%), hexadecane,2,6,10,14-tetramethyl (2.24%), nonadecane (2.50%), hexadecanoic acid, methyl ester (3.32%), hexadecanoic acid, ethyl ester (5.21%), heneicosane (3.06%), 9-octadecenoic acid, methyl ester (4.30%), phytol (15.37%), ethyl 9,12,15-octadecatrienoate (2.85%), eicosane (2.71%), squalene (9.31, 2.54, 6.57%), tricosane (1.45%), tetracosane (1.34%), docosane (1.33%), and alpha-tocopherol (6.17%).

Hexadecanoic acid identified in the hexane fraction has been reported to have antibacterial, antioxidant, and antitumor properties [35]. Furthermore, the ethanolic extract revealed the presence of caryophyllene (0.99%; the first compound with the least RT of 10.9 minutes) as shown in Table 3. The previous studies have reported α-caryophyllene and β-caryophyllene as anticancer essential oil components [36]. Park et al. [37] also reported the anti-tumorigenic potential of caryophyllene where he identified the down-regulation of vascular endothelial growth factor as the mechanism of action of caryophyllene. Phytol; the prominent compound identified in the hexane fraction has been reported to have antibacterial, antioxidant, and antitumor properties [35].

Fig. 2: Effect of Viburnum opulus extract on liver function parameters in albino Wistar rats after 28-day treatment, (a) Alanine aminotransferase, (b) aspartate aminotransferase, (c) alkaline phosphatase, (d) albumin, (e) urea, (f) total bilirubin, (g) direct bilirubin, (h) total protein. Values presented as mean±standard error of mean of 7 replicates. p<0.05 are accounted as statistically significant in comparison with the control group.

Fig. 3: The outcome of Viburnum opulus extract on the lipid profile of albino Wistar rats, (a) Low-density lipoprotein, (b) total cholesterol, (c) triglyceride, (d) high-density lipoprotein. Values represent mean±standard error of mean of 7 replicates. *p<0.05 are accounted as statistically significant in comparison with the control group.
Table 3: Phytochemical components of the ethanolic leaf extract of *V. opulus* (L.) as revealed by GC-MS spectra

| S.No. | Compound name                                                                 | Area percentage | RT (minutes) | Structure       |
|-------|-------------------------------------------------------------------------------|-----------------|--------------|-----------------|
| 1     | Caryophyllene                                                                 | 0.99            | 10.914       |                 |
| 2     | 1,2,3,5,6,8-hexahydro-4,7-dimethyl-1-(1-methylethyl)-(1S-cis)                 | 1.37            | 13.129       |                 |
| 3     | Germaciene D                                                                  | 1.20            | 12.242       |                 |
| 4     | Diethyl phthalate                                                             | 0.17            | 14.868       |                 |
| 5     | Neophytadiene                                                                 | 0.53            | 19.143       |                 |
| 6     | Hexadecanoic acid, ethyl ester                                                | 2.18            | 31.027       |                 |
| 7     | Ethyl 14-methyl-hexadecanoate                                                 | 0.61            | 23.474       |                 |
| 8     | Phytol                                                                       | 58.55           | 23.880       |                 |
| 9     | Linoleic acid ethyl ester                                                     | 2.02            | 24.533       |                 |
| 10    | 9,12,15-octadecatrienoic acid, ethyl ester (Z, Z, Z)                          | 12.62           | 24.636       |                 |
| 11    | Octadecanoic acid ethyl ester                                                 | 4.65            | 25.036       |                 |
| 12    | Squalene                                                                      | 0.02            | 25.797       |                 |
| 13    | Eicosanoic acid ethyl ester                                                   | 1.42            | 27.943       |                 |
| 14    | Docosanoic acid ethyl ester                                                   | 2.18            | 31.027       |                 |

RT: Retention time

antioxidant effect [38]. The ethanolic extract of *V. opulus* did not create any gross toxicological indications or death. Urea is used as a marker for kidney toxicity; as the glomerular damage is indicated by increased blood urea level which is often metabolized by the liver in urea cycle. However, there was no significant alteration in the urea level (Fig. 2e). No variation in the serum urea level which indicates the proper functioning of the kidneys. It is well known that liver damage is normally determined by the level of serum transaminases especially ALT. Certainly, non alteration in the serum ALT, AST, bilirubin, and protein is indicative of a lack of liver dysfunction and/or damage. These results were furthermore confirmed by the histological studies on the liver tissues where no degeneration of the liver tissues was observed (Fig. 4). The increase in albumin levels seen in the 500, 1000, and 1500 mg/kg bw groups may constitute a high risk of colloid osmotic blood pressure at increased doses of the plant extract. However, since albumin is important in determining the stability of the glomerular membrane and assessing the extremity of the disease associated with it [39], elevated levels observed in the treated groups may indicate improvement or increase in the liver function as increased albumin is essentially due to high liver synthetic ability [40]. HDL levels were significantly elevated (p<0.05) however reduction in LDL-cholesterol and triglyceride levels were observed. This indicates that the extract may have some potential effect to minimize cardiovascular risk factors which can otherwise lead to death [41]. Therefore, the extract of *V. opulus* is relatively harmless while the presence of various bio-active compounds detected after GC-MS analysis using the ethanolic extract of *V. opulus* accounts for the various uses by the traditional practitioner.

CONCLUSION

The GC-MS analysis suggests that the ethanolic leaf extract of *V. opulus* contains a wild range of fatty acids, heterocyclic compound with diverse antimicrobial properties and it is not toxic to rats within the dosage limit investigated.

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