Syzygium aqueum: A Polyphenol-Rich Leaf Extract Exhibits Antioxidant, Hepatoprotective, Pain-Killing and Anti-inflammatory Activities in Animal Models

Mansour Sobeh1*, Mona F. Mahmoud2, Ganna Petruk3, Samar Rezq2, Mohamed L. Ashour3, Fadia S. Youssef4, Assem M. El-Shazly4, Daria M. Monti3, Ashraf B. Abdel-Naim6 and Michael Wink1*

1 Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Heidelberg, Germany; 2 Department of Pharmacology and Toxicology, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt; 3 Department of Chemical Sciences, University of Naples Federico II, Naples, Italy; 4 Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University Abbassia, Cairo, Egypt; 5 Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt; 6 Department of Pharmacology and Toxicology, Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia

Syzygium aqueum is widely used in folk medicine. A polyphenol-rich extract from its leaves demonstrated a plethora of substantial pharmacological properties. The extract showed solid antioxidant properties in vitro and protected human keratinocytes (HaCaT cells) against UVA damage. The extract also reduced the elevated levels of ALT, AST, total bilirubin (TB), total cholesterol (TC) and triglycerides (TG) in rats with acute CCl4 intoxication. In addition to reducing the high MDA level, the extract noticeably restored GSH and SOD to the normal control levels in liver tissue homogenates and counteracted the deleterious histopathologic changes in liver after CCl4 injection. Additionally, the extract exhibited promising anti-inflammatory activities in vitro where it inhibited LOX, COX-1, and COX-2 with a higher COX-2 selectivity than that of indomethacin and diclofenac and reduced the extent of lysis of erythrocytes upon incubation with hypotonic buffer solution. S. aqueum extract also markedly reduced leukocyte numbers with similar activities to diclofenac in rats challenged with carrageenan. Additionally, administration of the extract abolished writhes induced by acetic acid in mice and prolonged the response latency in hot plate test. Meanwhile, the identified polyphenolics from the extract showed a certain affinity for the active pockets of 5-lipoxygenase (5-LOX), cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) explaining the observed anti-inflammatory activities. Finally, 87 secondary metabolites (mostly phenolics) were tentatively identified in the extract based on LC-MS/MS analyses. Syzygium aqueum displays good protection against oxidative stress, free radicals, and could be a good candidate for treating oxidative stress related diseases.

Keywords: Syzygium aqueum, polyphenols, antioxidant, hepatoprotective, pain-killing, anti-inflammatory
INTRODUCTION

Reactive oxygen species (ROS) are natural molecules in the body. ROS are formed by the immune system and through mitochondrial oxidative metabolism (aerobic respiration). Environmental stress through drugs, bacterial invasion, heat, heavy metal ions, and exposure to radiation and UV can strongly enhance ROS production (Finkel and Holbrook, 2000).

Reactive oxygen species and/or oxidative stress are able to cause damage to lipids, proteins, and DNA. At the DNA level, ROS can cause mutations of genes that can lead to malfunction of enzymes or regulatory proteins. Thus, ROS can be involved in the pathophysiology of several disorders, including cardiovascular diseases, hypertension, inflammation, cancer and neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease (van Wyk and Wink, 2015, 2017).

Elevated levels of ROS are involved in a number of physiological processes within the human body. Further to wound debridement, they also act as a fundamental host defense compounds and mediators of intracellular signaling cascade. ROS levels in the body are controlled by antioxidative molecules (glutathione) or enzymes (SOD, catalase). A functional interplay between ROS and antioxidants is essential in maintaining cell functions. However, ROS levels can be regulated by the uptake of exogenous antioxidants, among them natural antioxidant secondary metabolites, such as polyphenols, flavonoids, carotenoids, ascorbic acid, or allicin (van Wyk and Wink, 2015, 2017).

Several species belonging to the genus *Syzygium* have been extensively studied for their phytoconstituents as well as their biological activities; among them, *S. cumini*, *S. samarangense*, and *S. jambos*. The reported pharmacological activities include antioxidant, antiviral, anti-diabetic and hepatoprotective properties (Sobeh et al., 2018a,b).

The Water rose apple, *Syzygium aqueum*, a member of family Myrtaceae, is native to Indonesia and Malaysia but is presently widely distributed in the tropics. Several biologically active compounds have been isolated from the plant, among them, epigallocatechin, epigallocatechin gallate, vescalagin, castalagin, and samarangenins A and B (Nonaka et al., 1992). Several plant parts have been used in folk medicine. Substantial anti-hyperglycaemic activities were reported from the leaf extract and its individual components myricitrin, myrilgalone G and B, phloretin and europetin 3-O-rhamnoside from plants grown in Malaysia (Manaharan et al., 2012).

In this study, we investigated a methanol extract from *S. aqueum* leaves for its antioxidant activities in vitro and in keratinocytes (HaCaT cells). Also, the hepatoprotective activities were studied in a rat model against CCL₄-intoxicaton; in addition, the anti-inflammatory and antinociceptive activities were analyzed in rat and mouse models. Finally, the active secondary metabolites were characterized using HR-UPLC-MS/MS.
### TABLE 1 | Secondary metabolites from a methanol extract of *S. aqueum* leaves by HR-LC-MS/MS.

| No. | t<sub>r</sub> (min) | [M-H]− | MS/MS fragment | Tentatively identified compounds |
|-----|---------------------|--------|----------------|--------------------------------|
| 1   | 1.63                | 191    | 127            | Quinic acid<sup>a</sup>          |
| 2   | 2.23                | 341    | 179            | Caffeoyl hexoside                |
| 3   | 2.87                | 343    | 191            | Galloylquinic acid<sup>a</sup>   |
| 4   | 3.02                | 331    | 169            | Galloyl hexose<sup>a</sup>       |
| 5   | 3.26                | 609    | 305, 423, 441, 591 | (epi)-Gallocatechin-(epi)-gallocatechin |
| 6   | 3.88                | 483    | 169, 331       | Ddigalloyl hexose                |
| 7   | 4.43                | 933    | 301, 631, 915  | Castalagin/Vescalagin<sup>−,a</sup> |
| 8   | 6.13                | 477    | 169, 313, 331  | Gallic acid coumaroyl-hexose     |
| 9   | 6.28                | 633    | 301, 463, 615  | Galloyl-hexahydroxydiphenoyl (HHDP)-hexoside |
| 10  | 6.36                | 609    | 305, 423, 441  | (epi)-Gallocatechin-(epi)-gallocatechin<sup>b</sup> |
| 11  | 6.51                | 343    | 125, 169       | Galloylquinic acid               |
| 12  | 6.76                | 1583   | 765, 935, 1537, 1565 | Eugeniflorin D2<sup>a</sup> |
| 13  | 7.85                | 305    | 125, 179       | (epi)-Gallocatechin<sup>−,a</sup> |
| 14  | 9.14                | 179    | 161            | Caffeic acid                     |
| 15  | 9.27                | 315    | 153            | p-Coumaric acid hexoside         |
| 16  | 10.29               | 441    | 179, 271, 423  | (epi)-Catechin galate            |
| 17  | 12.23               | 301    | 229, 257, 301  | Ellagic acid<sup>b</sup>         |
| 18  | 12.87               | 593    | 289, 425, 557  | (epi)-Catechin-(epi)-gallocatechin |
| 19  | 16.12               | 761    | 305, 423, 593, 609 | (epi)-Gallocatechin-(epi)-gallocatechin galate<sup>b</sup> |
| 20  | 17.11               | 897    | 305, 559, 609, 771 | (epi)-Gallocatechin-(epi)-gallocatechin-(epi)-catechin |
| 21  | 17.81               | 783    | 301, 615, 765  | Pedunculagin<sup>+</sup>         |
| 22  | 18.21               | 273    | 187, 229       | (epi)-Arzelechin                 |
| 23  | 19.90               | 633    | 301, 481, 615  | Galloyl-hexahydroxydiphenoyl (HHDP)-hexoside |
| 24  | 21.20               | 745    | 289, 577, 593, 727 | (epi)-Catechin-(epi)-gallocatechin galate<sup>a</sup> |
| 25  | 22.61               | 285    | 153            | p-Coumaric acid pentoside        |
| 26  | 22.72               | 483    | 169, 331       | Digalloyl hexose                 |
| 27  | 23.41               | 759    | 229, 301, 633  | Samarangenin A<sup>+</sup>       |
| 28  | 24.79               | 577    | 289, 407, 425, 559 | (epi)-Catechin-(epi)-catechin<sup>a</sup> |
| 29  | 25.74               | 745    | 305, 423, 457, 593 | (epi)-Gallocatechin-(epi)-catechin galate<sup>a</sup> |
| 30  | 25.94               | 575    | 287, 423       | (epi)-Catechin-A-(epi)-catechin |
| 31  | 28.25               | 761    | 305, 423, 609  | (epi)-Gallocatechin-(epi)-gallocatechin galate |
| 32  | 28.91               | 785    | 301, 483, 633, 765 | Digalloyl- HHDP-hexoside |
| 33  | 29.89               | 457    | 305, 331       | (epi)-Gallocatechin galate<sup>−,a, y</sup> |
| 34  | 33.48               | 745    | 289, 441, 593, 619 | (epi)-Gallocatechin-(epi)-catechin galate<sup>a</sup> |
| 35  | 33.7                | 1217   | 423, 761, 1047, 1065 | (epi)-Gallocatechin-(epi)-gallocatechin-(epi)-gallocatechin<sup>a</sup> |
| 36  | 34.92               | 1049   | 423, 745, 761  | (epi)-Catechin-(epi)-gallocatechin - (epi)-gallocatechin galate<sup>a</sup> |
| 37  | 35.94               | 785    | 301, 483, 615, 763, 765 | Digalloyl-HHDP-hexoside |
| 38  | 37.00               | 1201   | 423, 475, 1049 | (epi)-Catechin-(epi)-gallocatechin-(epi)-gallocatechin-(epi)-gallocatechin galate |
| 39  | 37.27               | 367    | 191            | Feruloylquinic acid             |
| 40  | 37.5                | 337    | 191            | p-Coumaroylquinic acid<sup>a</sup> |
| 41  | 38.82               | 881    | 423, 591, 729  | (epi)-catechin galate-(epi)-catechin galate |
| 42  | 40.71               | 1217   | 423, 761, 913, 1030 | (epi)-Gallocatechin-(epi)-gallocatechin-(epi)-gallocatechin-(epi)-gallocatechin<sup>a</sup> |
| 43  | 41.09               | 441    | 169, 289       | (epi)-Catechin galate<sup>−,a</sup> |
| 44  | 42.18               | 1017   | 407, 575, 729  | (epi)-Catechin-(epi)-catechin-(epi)-catechin galate<sup>a</sup> |
| 45  | 42.23               | 913    | 423, 591, 761  | Prodelphinidin B2 3,3′-digalate<sup>+</sup> |
| 46  | 43.13               | 451    | 169, 289       | (epi)catechin glycoside         |
| 47  | 45.41               | 911    | 285, 423, 571, 759 | Samarangenin B<sup>+</sup> |
| 48  | 49.03               | 625    | 317, 463       | Myricetin rhamnosyl-hexoside<sup>a</sup> |
| 49  | 49.53               | 479    | 317            | Myricetin hexoside<sup>a</sup>   |
| 50  | 50.27               | 449    | 179, 317       | Myricetin pentoside<sup>a</sup>  |
| 51  | 51.72               | 595    | 179, 271, 301, 463 | Quercetin pentosyl-hexoside |
| 52  | 52.08               | 623    | 286, 447       | Myrigalone-G glucuronide-hexoside |

(Continued)
TABLE 1 | Continued

| No. | t_R (min) | [M-H]^− | MS/MS fragment | Tentatively identified compounds |
|-----|-----------|---------|----------------|---------------------------------|
| 53  | 53.15     | 463     | 179, 317       | Myricetin-O-rhamnoside^a,*¥      |
| 54  | 54.43     | 375     | 361            | Methyligstroside aglycone        |
| 55  | 54.83     | 631     | 317, 479       | Myricetin galloy-hexoside        |
| 56  | 56.85     | 729     | 289, 407, 559  | Procyanidin dimer monogallate^a  |
| 57  | 57.15     | 593     | 285, 447       | Myrigalone-G-rhamnosyl-hexoside  |
| 58  | 57.22     | 601     | 317, 449       | Myricetin galloy-pentoside       |
| 59  | 57.9      | 881     | 289, 407, 559, 729 | (epi)-Catechin galloy-(epi)-catechin gallate^b |
| 60  | 58.08     | 493     | 331            | Europetin hexoside              |
| 61  | 58.18     | 609     | 301            | Quercetin rutinoside^a           |
| 62  | 58.33     | 645     | 331, 493       | Europetin galloy-hexoside        |
| 63  | 58.51     | 737     | 300, 433       | Quercetin pentosyl digallate     |
| 64  | 58.58     | 749     | 301, 447, 595, 597 | Quercetin rhamnosyl-digallate     |
| 65  | 62.17     | 433     | 301            | Quercetin pentoside              |
| 66  | 63.36     | 477     | 331            | Europetin rhamnoside^e           |
| 67  | 63.84     | 447     | 301            | Quercetin rhamnoside             |
| 68  | 64.04     | 615     | 317, 463       | Myricetin galloy-rhamnoside^a,c  |
| 69  | 64.13     | 615     | 301, 463       | Quercetin galloy-hexoside^a      |
| 70  | 64.8      | 447     | 315            | Isorhamnetin pentoside           |
| 71  | 64.98     | 417     | 285            | Myrigalone-G-pentoside           |
| 72  | 65.28     | 461     | 315            | Isorhamnetin rhamnoside          |
| 73  | 65.3      | 585     | 301, 433       | Quercetin galloy-pentoside^a      |
| 74  | 66.34     | 601     | 179, 317, 449  | Myrigalone-G-pentoside           |
| 75  | 66.43     | 935     | 301, 463, 633, 783 | Quercetin hexose trigallate      |
| 76  | 67.01     | 599     | 301, 447       | Quercetin galloy-rhamnoside      |
| 77  | 67.36     | 615     | 317, 463       | Myricetin galloy-rhamnoside^a     |
| 78  | 68.06     | 417     | 285            | Myrigalone-G-pentoside           |
| 79  | 68.15     | 523     | 361            | Ligrostoside                     |
| 80  | 68.26     | 431     | 285            | Myrigalone-G-rhamnoside          |
| 81  | 68.27     | 329     | 285            | Acetylmeryrigalone-G^*            |
| 82  | 69.09     | 461     | 329            | Tricin pentoside                 |
| 83  | 69.35     | 585     | 179, 301, 433  | Quercetin galloy-pentoside       |
| 84  | 71.56     | 629     | 179, 301, 463, 477 | Quercetin methyl galloy-hexoside |
| 85  | 72.11     | 299     | 151, 243, 271  | Myrigalone-B^*                   |
| 86  | 73.36     | 285     | 163, 241, 271  | Myrigalone-G^*                   |
| 87  | 74.20     | 269     | 121, 255, 271  | Cryptostrobin^a                   |

^aPreviously isolated from the plant (Nonaka et al., 1992) and (Manaharan et al., 2012). ¥Identification was confirmed using authentic standard compounds. aIdentification was based on Sobeh et al. (2018b). ¥Identification was based on Sobeh et al. (2018a).

MATERIALS AND METHODS

Plant Material and Extraction

The leaves of *S. aqueum* were collected from trees grown in private garden, Egypt. The species was identified by Mrs. Therese Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and El-Orman Botanical Garden, Giza, Egypt. A voucher specimen (accession number: PHG-P-SA-181) was deposited at Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University. The air-dried and milled leaf powder (100 g) was extracted with 100% methanol at ambient temperature (3 × 500 mL). The whole mixture was filtered and concentrated under vacuum at 40°C giving a semisolid residue. The latter was frozen at −70°C and lyophilized for 72 h yielding fine dried powder (12 g).

LC–HRESI–MS–MS

An HPLC Agilent 1200 series instrument was used to analyze the sample. A Gemini 3 μm C18 110 Å column from Phenomenex with dimensions 100 × 1 mm i.d., protected with RP C18 100 Å guard column with dimensions (5 mm × 300 μm i.d., 5 μm) was used. The mobile phase was (A) 2% acetic acid and (B) 90% MeOH, 2% acetic acid at a flow rate of 50 μL/min. The gradient was from 5% B at 0 min to 50% B in 50 min and then increased to 90% in 10 min and kept for 5 min. Fourier transform ion cyclotron resonance mass analyzer was used equipped with an electrospray ionization (ESI) system. The system was controlled using X-calibur® software. The data were collected in the negative ion mode as described before (Sobeh et al., 2018b). The full mass scan covered the mass range from 150 to 2000 m/z with resolution up to 100000.
Table 2 | Antioxidant properties of the extract as compared to positive controls EGCG and vitamin C.

| Assay                        | S. aqueum extract | EGCG | Ascorbic acid |
|------------------------------|-------------------|------|---------------|
| DPPH (EC50 µg/mL)            | 6.80 ± 0.15       | 3.50 ± 0.23 | 2.95 ± 0.13   |
| TEAC (Trolox equivalents/mg of sample) | 2073 ± 17 | 5293 ± 23 | -             |
| FRAP (Fe²⁺ equivalents/mg of sample) | 11.51 ± 0.82 | 25.23 ± 1.32 | -             |
| TAC (Total antioxidant capacity, U/L) | 18.72 ± 1.11 | - | 26.41 ± 1.75 |

Figure 2 | Effect of Syzygium aqueum extracts on human keratinocytes. (A) Biocompatibility of S. aqueum extract in dose (25–200 µg/mL) and time (24–48 h) manner. Survival rate of the cells was assessed by the MTT assay. (B) ROS production in cells treated or untreated with the extract (100 µg/mL) and then irradiated by 100 J/cm² UVA (+) or not (-). (C) Total GSH levels determined by DTNB assay. Results expressed as mean ± SD of three assays. Significant ∗p < 0.01; ∗∗p < 0.001 compared to untreated control. Analysis performed by two-way ANOVA followed by Bonferroni’s multiple comparisons test. (D) Immunoblotting bands of the oxidative stress activated protein, p38. An equal amount (100 µg) of total lysate from each sample was resolved by SDS-PAGE with GAPDH as a control.

Biological Activity

Antioxidant Activities in Vitro

Total phenolic contents were determined using the Folin-Ciocalteu method and the antioxidant activities were investigated by DPPH radical scavenging activity, FRAP assay and ABTS assay, as previously described (Ghareeb et al., 2017).

Total Antioxidant Capacity (TAC) Assay

Total antioxidant capacity was assessed using a commercially available TAC ELISA kit (MBS726896, MyBioSource, Inc., San Diego, CA, United States) according to the manufacturer’s instructions using ascorbic acid as the reference standard. Briefly, the extract, ascorbic acid or PBS were incubated with TAC-HRP conjugate in pre-coated plate for 1 h. This was followed by proper washing and incubation with a substrate for HRP enzyme. A yellow color was formed which is inversely proportional to the TAC concentration. After 30 min, the stop solution was added to terminate the reaction. The intensity of the yellow color formed was measured at 450 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, United States). A standard curve was established using serial dilutions of the standard. The sample activity (U/L) was calculated from the standard curve equation.

Cell Culture and MTT Assay

Human epidermal keratinocytes (HaCaT), provided by Innoprot (Biscay, Spain), were cultured as described in Petruk et al. (2016). For dose and time dependent biocompatibility experiments, cells were seeded in 96-well plates at a density of 2 × 10⁴ cells/well. Twenty four hour after seeding, increasing concentrations of the methanol extract (from 25 to 200 µg/mL) were added to the cells for 24 and 48 h. Cell viability was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described in Monti et al. (2015). Cell survival was expressed as the percentage of viable cells in the presence of the extract compared to controls. Two groups of cells were used as control, i.e., untreated cells and cells supplemented with identical volumes of buffer. Each sample was tested in three independent analyses, each carried out in triplicates.

Oxidative Stress in HaCaT Cells

To investigate oxidative stress, HaCaT cells were plated at a density of 2 × 10⁴ cells/cm². Twenty four hour after seeding, cells were incubated for 2 h in the presence or absence of 100 µg/mL of the extract and then exposed to UVA (100 J/cm²). Then, ROS levels, intracellular GSH levels and the phosphorylation of p38 were analyzed as described in Petruk et al. (2016). The other
assays namely DCFDA assay, DTNB assay and Western blot analyses were performed as described in Petruk et al. (2016).

**Hepatoprotective Activity in Vivo**

**Animals and experimental design**

Male Sprague-Dawley rats, weighing 200–250 g, were purchased from the animal resource branch, King Abdulaziz University, Geddah (KSA). The rats were fed on rodent chow and water ad libitum and housed in conventional cages at 22 ± 2°C, with a 12 h light–dark cycle. The study protocol was approved by the Unit of Biomedical Ethics Research Committee, Faculty of Medicine, King Abdulaziz University, following the Institutional Animal Care and Use Committee guidelines (Reference # 157-14). In brief, 24 rats were divided into four groups. Group (1) served as a control and obtained water orally followed by intraperitoneal (i.p.) injection of corn oil after 4 h. Group (2) was injected a single dose of CCl₄-corn oil (Sigma–Aldrich, St. Louis, MO, United States) (1 ml/kg, of 50% mixture). Group (3) served as a positive control and was administered the known hepatoprotective silymarin (200 mg/kg orally). Group (4) was pretreated with *S. aqueum* extract (200 mg/kg orally). Groups (3 and 4) were injected CCl₄-corn oil (1 ml/kg, of 50% mixture, i.p.) after 4 h from the pretreatment (Sobeh et al., 2018a).

Twenty four hour after the treatments, the rats were anesthetized, and blood samples were collected by cardiac puncture, allowed to clot, centrifuged for 10 min at 2113 × g, and then kept at −80°C until analysis. Finally, the rats were sacrificed, and liver tissues were dissected. The liver tissues were cut and fixed in 10% formalin/saline and embedded in paraffin for histopathological investigations. The remaining liver tissues were re-weighed, washed and homogenized in ice-cold PBS to yield 10% w/v homogenates and then stored at −80°C until analyses.
Liver biomarkers
Mindray BS-120 clinical chemistry auto-analyzer (Shenzhen Mindray Bio-Medical Electronics Co. Ltd., Shenzhen, China) was used to determine the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), total cholesterol (TC), and triglycerides (TG). The levels of oxidative stress markers glutathione (GSH), lipid peroxidation marker malondialdehyde (MDA) and the activity of the antioxidant enzyme superoxide dismutase (SOD) were quantified utilizing the commercially available kits (Biodiagnostics, Cairo, Egypt).

Histopathological examination
Liver tissue samples were stored in 10% buffered neutral formalin for 24 h and then washed with tap water. Dehydration was conducted using serial dilutions of methyl, ethyl and absolute ethyl alcohols. The tissue sections were then embedded in xylene, immersed in paraffin, and dried inside hot air oven at 56°C for 24 h. Tissue sections, 4 µm thickness, were prepared using slide microtome, placed on glass slides, and stained with eosin and hematoxylin. The latter was investigated using the light electric microscope (Olympus BX-50 Olympus Corporation, Tokyo, Japan) as described in Sobeh et al. (2018a).

Anti-inflammatory Activities
In Vitro Anti-inflammatory activities
The capacity of the extract to inhibit lipoxygenase was determined using a lipoxygenase inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI, United States) according to the manufacturer’s instruction and reported studies (Abdelall et al., 2016). The data are expressed as IC_{50} value, which is the concentration causing 50% enzyme inhibition (IC_{50}). Furthermore, the COX-2 selectivity index (SI values) which is defined as IC_{50} (COX-1)/IC_{50} (COX-2) was calculated and compared to that of celecoxib, indomethacin, and diclofenac which were used as reference standards.

Membrane stabilizing activity (hypotonic solution-induced hemolysis)
The membrane stabilizing activity of the extract was assessed in erythrocytes; hemolysis was induced by osmotic shock (Shinde et al., 1999). Fresh whole blood (10 mL) was collected from a healthy human volunteer who had not taken any NSAIDS for 2 weeks prior to the experiment. This was followed by centrifugation of heparinized blood (2113 × g/10 min) and washing with normal saline. Next, a 40% (v/v) RBCs suspension in 10 mM sodium phosphate buffer solution (NaH_{2}PO_{4}, 2H_{2}O, 0.26 g; Na_{2}HPO_{4}, 1.15 g; NaCl, 9 g) was prepared. A 0.5 mL of the previous suspension was incubated for 10 min with 5 mL of hypotonic solution (50 mM NaCl in 10 mM sodium phosphate buffered saline, pH 7.4) containing with the extract (1000 µg/mL), aspirin or diclofenac (100 µg/mL) as reference drugs. The samples were then centrifuged at 3000 g for 10 min. The absorbance of the supernatant was then detected at 540 nm. The ability of the tested sample to inhibit RBCs hemolysis was calculated using the following equation:

\[
\text{Hemolysis inhibition } \% = \left( \frac{OD1 - OD2}{OD1} \right) \times 100
\]
where OD1 = Optical Density of the control (cells incubated with the buffer only) and OD2 = Optical Density of test sample.

**Carrageenan-induced hind-paw edema**

Right hind paw edema was induced in rats by injecting freshly prepared carrageenan solution (1% in 0.9% NaCl, 0.1 mL), into the sub plantar tissue. 1 h earlier, the vehicle (10 mL/kg), S. aqueum extract (300 mg/kg, p.o.) or diclofenac (10 mg/kg) were given orally. The paw thickness (mm) was measured in the dorsal- plantar axis by a caliper ruler before and after the carrageenan injection at hourly intervals for 6 h and then at 24 h. The cumulative anti-inflammatory effect during the whole observation period (0–24 h) was estimated by calculating the area under changes in paw thickness-time curve (AUC0–24).

**TABLE 3** | Inhibition activities of the extract on LOX, COX-1, and COX-2 enzymes.

| Treatment            | IC50 (µg/mL) | IC50 (µg/mL) | IC50 (µg/mL) | IC50 (µg/mL) |
|----------------------|--------------|--------------|--------------|--------------|
|                      | LOX          | COX-1        | COX-2        | SI           |
| S. aqueum extract    | 2.54 ± 0.19  | 7.11 ± 0.43  | 0.12 ± 0.005 | 59.3         |
| Celecoxib            | –            | 15.1 ± 0.72  | 0.049 ± 0.002| 308.2        |
| Diclofenac           | 2.11 ± 0.14  | 3.8 ± 0.17   | 0.84 ± 0.04  | 4.5          |
| Indomethacin         | –            | 0.041 ± 0.001| 0.51 ± 0.02  | 0.08         |
| Zileuton             | 3.51 ± 0.21  | –            | –            | –            |

SI is COX selectivity index which is defined as IC50 (COX-1)/IC50 (COX-2).

**Recruitment of leukocyte to peritoneal cavity in mice**

The recruitment of leukocytes to the peritoneal cavity was assessed as described previously (Silva-Comar et al., 2014). Briefly, Swiss albino mice (n = 5–8/group), were orally treated with the vehicle (1 mL/100 g, p.o.) or S. aqueum extract (300 mg/kg) 30 min before the intraperitoneal injection of 0.1 mL carrageenan solution (500 µg/mice) or 0.1 mL sterile saline. Diclofenac (10 mg/kg, p.o.) was used as the reference anti-inflammatory drug. The animals were euthanized 3 h later and the peritoneal cavity was washed with 3 mL of phosphate-buffered saline (PBS) contained 1 mM ethylenediaminetetraacetic acid (EDTA). The total leukocyte count was determined in the peritoneal cavity wash using a hemocytometer and expressed as number of cells/mL.

**Antinociceptive Activity**

**Acetic acid-induced abdominal writhing**

The peripheral analgesic activity of the extract was evaluated using acetic acid induced writhing model in mice (Nakamura et al., 1986). The animals were divided into three groups (5–7 mice). Group (1) received the vehicle (1% Tween 80, 10 mL/kg) and served as negative control. Group (2) received the extract (300 mg/kg, p.o.) and group (3) received diclofenac (10 mg/kg, a reference drug) 1 h prior i.p. injection of 0.7% acetic acid (1 mL/100 g). The number of writhes (contraction of abdomen, turning of trunk and extension of hind legs) was observed for 25 min.

**Hot plate test**

The hot plate test was carried out to test a possible central analgesic activity of the extract (Macdonald et al., 1946; Eddy and Leimbach, 1953). Mice were divided in different groups (5 each) and receive either the extract (300 mg/kg, p.o.) or the vehicle (10 mL/kg, p.o.). Another group of mice received the opioid analgesic nalbuphine as reference central analgesic. After 60 min, the mice were individually placed in a hot plate heated at 55 ± 1°C and the animal was studied for any sign of response to heat-induced nociceptive pain (licking of the fore and hind paws, hind paw lifting or jumping). The latency until mice showed the first signs of discomfort was recorded before (baseline) and at 1, 2, 3, and 4 h following different treatments.

**Molecular Modeling**

In silico virtual screening studies of the major constituents identified in the bioactive methanol extract of S. aqueum was performed using the C-docker protocol on 5-lipoxygenase (PDB ID 3V99, 2.48 Å), cyclooxygenase-I (PDB ID 2OYE, 2.85 Å) and cyclooxygenase-II (PDB ID 3V99, 2.48 Å), cyclooxygenase-I (PDB ID 2OYE, 2.85 Å) and 3,4, and 5-lipoxygenase (PDB ID 3V99, 2.48 Å).
FIGURE 6 | Anti-inflammatory activities of S. aqueum leaf extract. (A) Effect of 1 h prior administration of S. aqueum (300 mg/kg, p.o.) or diclofenac (10 mg/kg, p.o.) on carrageenan (1% suspension, 0.1 ml/rat) induced-hind paw edema in rats. Edema thickness (mm) was measured before and then hourly for 6 h and at 24 h after carrageenan injection. Data represents the AUC0-24 and are expressed as mean ± SEM (n = 5–7). (B) Effect of carrageenan (500 µg, i.p.) on leukocytes migration into the peritoneal cavity (total number × 10⁶) with or without 1 h prior treatment with the extract (300 mg/kg, p.o.) or diclofenac (10 mg/kg, p.o.). Data are expressed as mean ± SEM (n = 5–7). *p < 0.01 vs. control values; #p < 0.05 vs. carrageenan.

FIGURE 7 | Antinociceptive activities of S. aqueum leaf extract. (A) Effect of S. aqueum extract (300 mg/kg, p.o.) or diclofenac (10 mg/kg, p.o.) on acetic acid-induced writhing (0.7%, 1 mL/100 g) in mice. (B) Hot plate response latency measured 1–4 h after vehicle, extract (300 mg/kg, p.o.) or nalbuphine (10 mg/kg, p.o.) administration in mice. Data is expressed as mean ± SEM (n = 5–7). *p < 0.001 vs. control values.

cyclooxygenase-II (PDB ID 3LN1, 2.3 Å) that were downloaded from protein data bank1. This was done by applying both pH and rule-based ionization methods for the preparation of ligands with Discovery Studio 2.5 (Accelrys Inc., San Diego, CA, United States). In silico virtual screening was performed within the active pocket of the previously mentioned enzymes and the free binding energies for the highly stable docking poses were calculated as previously discussed (Sobeh et al., 2016; Ashour et al., 2017; Youssef et al., 2017).

Data Analysis

Data were analyzed using GraphPad Prism software, version 5.00 (GraphPad Software, Inc., La Jolla, CA, United States). Analysis of Variance (ANOVA) or repeated-measures analysis of variance (RM-ANOVA) followed by Tukey’s post hoc test and Student’s t-test were used to state differences between groups. Otherwise mentioned, data are expressed as mean ± SEM.

RESULTS

Secondary Metabolites From S. aqueum

Utilizing high resolution LC-ESI-MS/MS, 87 compounds were characterized in a methanol extract of S. aqueum leaves. Flavonoids, represented by myricetin rhamnoside (53), myrigalone-G pentoside (71), quercetin galloyl-pentoside (73), cryptostrobin (87), myrigalone-B (85), and myrigalone-G (86) dominated the extract, in addition, proanthocyanidins with 1www.pdb.org
mainly samarangenin A (27), (epi)-gallocatechin gallate (33), and (epi)-catechin-(epi)-gallocatechin-(epi)-gallocatechin gallate (36) were abundant. Also, digalloyl-hexahydroxydiphenoyl (HHDP)-hexoside (37), as an example for ellagitannins, was among the major constituents. Some phenolic acid derivatives and other ellagitannins were characterized as well (Figure 1 and Table 1). Representative MS/MS fragmentation of some identified compounds are presented in Supplementary Figures S1–S45.

Antioxidant Activities in Vitro and in Keratinocytes

Initially, we evaluated the antioxidant potential of the leaf extract in vitro using DPPH, TEAC and FRAP assays as well as total antioxidant capacity (TAC), as reported in Table 2. The total phenolic content amounted to 453 mg GAE/g extract. The extract demonstrated substantial activities in all assays. (epi)Gallocatechin gallate (EGCG) and ascorbic acid were used as reference compounds.

We also investigated the antioxidant capacity of the extract in human keratinocytes. First, we tested the biocompatibility of the extract in HaCaT cells by a time-course and dose-response test, using increasing amount of extract (from 25 to 200 µg/mL). As shown in Figure 2A, cell viability was not significantly affected at any of the concentration and time analyzed. Then, we tested the extract in HaCaT cells by a time-course and dose-response test, using increasing amount of extract (from 25 to 200 µg/mL). As shown in Figure 2A, cell viability was not significantly affected at any of the concentration and time analyzed. Then, we investigated whether the extract had protective properties against oxidative stress. In all the experiments, cells were pre-treated with the extract (100 µg/mL) for 2 h before challenging cells with UVA irradiation to induce oxidative stress (100 J/cm²). At the end of the incubation, intracellular ROS levels were analyzed by using H2DCF-DA (2,7-dichlorofluorescin diacetate) (Figure 2B). It is apparent that UVA significantly increased DCF fluorescence intensity, compared to the non-irradiated cells. However, pre-treatment of the cells with the extract showed reduced ROS levels. The UVA induced increase in ROS levels is normally followed by a decrease in total GSH levels. In fact, as shown in Figure 2C, cells irradiated by UVA have dramatically reduced GSH levels. As expected, cells pre-treated with the extract and then exposed to oxidative stress showed unaltered GSH levels, compared to the untreated cells. The ability of the extract to protect HaCaT cells from UVA damages was confirmed by Western blot analysis. In particular, a mitogen activated protein kinase (p38) phosphorylation levels were analyzed (Figure 2D). We found that treatment of cells with extract does not activate this protein. Instead, as expected, UVA induced a significant increase in the phosphorylation levels of p38. On the other hand, when cells were pre-incubated with the extract and then exposed to UVA radiations, the phosphorylation levels of p38 were significantly lower to that observed in irradiated cells.

Hepatoprotective Activities

In order to validate the in vitro and cell culture results, we tested the antioxidant activity of the extract in vivo. Thus, the preventive action of the extract against the intoxication of CCl4 in rats was determined. CCl4 administration elevated all markers for liver damage. When the rats were treated with the extract prior CCl4 injection, protective activities were observed in all liver parameters. The extract (200 mg/kg b.w.) significantly reduced the elevated levels of ALT, AST, TB, TC, and TG, comparable to activities of the standard lignin from Silybum marianum, silymarin, Figure 3. Also, CCl4 injection significantly decreased the levels of GSH and SOD and increased MDA level in liver tissue homogenates. The extract markedly restored their levels to the normal control levels except for MDA, Figure 4. The hepatoprotection through the leaf extract was then further evidenced using a histopathologic study. The extract protected the liver against the deleterious effect of CCl4, Figure 5.

Anti-inflammatory Activities

Besides the antioxidant and hepatoprotective activity of the extract, we investigated its anti-inflammatory activity. In particular, we examined the ability of the extract to inhibit lipoxygenase (LOX) and cyclooxygenases (COX1/2). Interestingly, the extract inhibited LOX stronger than zileuton, the reference LOX inhibitor, as reported in Table 3. Additionally, the extract was able to inhibit both COX-1 and COX-2

| Compound               | 5-LOX          | COX-1          | COX-2          |
|------------------------|----------------|----------------|----------------|
|                        | Rule-based ionization | pH-based ionization | Rule-based ionization | pH-based ionization | Rule-based ionization | pH-based ionization |
| NDGA (lead)            | −44.49         | −65.44         | NT             | NT              | NT               | NT               |
| Diclofenac (lead)      | −30.07         | −34.64         | −40.06         | −40.72          | −40.14           | −40.74           |
| epiGallocatechin gallate | −61.15        | −56.22         | −64.67         | −66.33          | −61.24           | −67.13           |
| Cryptotrobin           | −31.58         | −45.63         | −40.80         | −44.04          | −39.60           | −47.04           |
| Europetin rhamnoside   | −54.14         | −58.23         | −61.41         | −63.90          | −56.53           | −66.52           |
| Myricetin rhamnoside   | −50.44         | −61.19         | −61.75         | −57.07          | −57.16           | −67.74           |
| Myrigaline B           | −30.89         | −31.62         | −37.01         | −37.83          | −37.03           | −41.87           |
| Myrigaline G           | −38.15         | −38.31         | −43.49         | −47.63          | −49.87           | −49.97           |
| Samarangenin A         | FD             | 58.70          | FD             | FD              | FD               | FD               |
| Epicatechin-epigallocatechin-epigallocatechin gallate | −123.93 | −123.62 | FD | FD | FD | FD |

NT, not tested. FD, fail to dock.
FIGURE 8: 2D and 3D binding mode of (A) epicatechin-epigallocatechin-epigallocatechin gallate in 5-LOX, (B) epigallocatechin gallate in COX-1, (C) myricetin rhamnoside in COX-2 active sites using molecular modeling experiments applying pH-based ionization method.
Moreover, the extract exhibited a SI value of 59.3, indicating higher selectivity toward COX-2 compared to COX-1. Noteworthy, the extract showed a higher COX-2 selectivity than that of indomethacin and diclofenac, but lower than that of celecoxib, as reported in Table 3.

In another experiment, we studied if the extract could counteract membrane disturbance in red blood cells (RBCs). At 1 mg/mL, the extract reduced the extent of RBCs lysis upon incubation with hypotonic buffer solution. Aspirin and diclofenac were used as reference drugs (Table 4).

We also explored the in vivo anti-inflammatory properties of the extract using the well characterized model, carrageenan-induced paw edema (Winter et al., 1962; Zhang et al., 2008). Rats pre-treated with the extract (300 mg/kg, p.o.) 1 h before sub-planter carrageenan injection (1% suspension, 0.1 mL/rat) significantly attenuated carrageenan-induced paw edema and decreased the AUC$_{0-24}$ value by 14.15%. The positive control, diclofenac, decreased AUC$_{0-24}$ value by 38.2%, as reported in Figure 6B. Similar activities were observed by a standard anti-inflammatory compound, diclofenac (10 mg/kg, p.o.).

**Antinociceptive and Analgesic Activities**

In addition to the aforementioned activities, we researched the antinociceptive and analgesic activities of the leaf extract in vivo against acetic acid-induced writhing. As shown in Figure 7A, oral pre-treatment with the extract (300 mg/kg) almost abolished (96% reduction) the writhes induced by acetic acid in mice when given 1 h earlier (p < 0.001) compared to control. Diclofenac, a reference analgesic drug, achieved 65% reduction of the control writhes, (p < 0.001). These activities were also confirmed using hot plate test in mice where animals pre-treated with the extract (300 mg/kg, p.o.) prolonged the response latency when measured at 1, 2, 3, and 4 h after administration. This effect was significant at 1 and 2 h (14 ± 2.8 and 14.6 ± 2.5 s, respectively) post-treatment when compared to the control group (3.8 ± 0.5 and 4.2 ± 0.58 s, respectively). The standard reference drug, (nalbuphine, 10 mg/kg, i.p.), significantly increased response latency all tested time points with the maximum effect achieved at the 2 h (17.5 ± 3.37 s) (Figure 7B).

**In Silico Virtual Screening**

In silico virtual screening studies were carried out on three crucial enzymes implicated in the occurrence and subsequent progression of inflammation namely, 5-lipoxygenase (5-LOX), cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Most of the docked compounds showed certain stability within the active pockets of all tested enzymes except for samarangenin A and epicatechin-epigallocatechin-epigallocatechin gallate that failed to dock in both COX-1 and COX-2 owing to the relatively larger size of the molecules comparable to the active pockets of target enzymes (Table 5).

Among all the docked compounds, epicatechin-epigallocatechin-epigallocatechin gallate revealed the greatest stability with a pronounced highest fitting in the active site of 5-LOX displaying free binding energies of $-123.62$ and $-123.93$ kcal/mol, in the pH-based and rule-based ionization methods, respectively. In this virtue, it highly exceeds that of nordihydroguataeric acid (NDGA), the potent well-known 5-LOX inhibitor that showed ΔG of $-65.44$ and $-44.49$ kcal/mol applying both previously mentioned methods, respectively. The apparent stability of epicatechin-epigallocatechin-epigallocatechin gallate in the active site of 5-LOX is strongly attributed to the formation of numerous firm hydrogen bonds with Ala 672, Asp 176, Gln557, Gln 363, Gln 409, Val 175, Val 671 and Thr 364 amino acid moieties in the active site of 5-LOX displaying free binding energies of $-64.67$ kcal/mol applying both rule-based ionization methods illustrated in Figure 8. Regarding COX-1, (epi)-gallocatechin gallate revealed the highest fitting as manifested from the free binding energies, which are $-66.33$ and $-64.67$ kcal/mol in the pH and rule-based ionization methods, respectively. This firm stability in the active pockets is mainly due to the formation of six stable hydrogen bonds with Arg 120, Gln 192, Ile 517, Tyr 355, Trp 387 Ser 530 in addition to a π-π interaction and ionic bond with Arg 120 as revealed from the pH-based ionization method (Figure 8).

Meanwhile, myricetin rhamnoside showed the highest fitting...
in COX-2 displaying free binding energies of \(-67.74\) kcal/mol, in the pH-based ionization method with \(-57.16\) kcal/mol in the rule-based ionization method. The tight fitting of myricetin rhamnoside in the active site of COX-2 could be explained in terms of the formation of four firm hydrogen bonds with Arg 106, Arg 499, Tyr 371 and Ser 333 amino acid residues existing at the active site in addition to the formation of a \(\pi-\pi\) bond with His 75 as illustrated from the pH-based ionization method (Figure 8). The presence of epicatechin-epigallocatechin-epigallocatechin gallate, (epi)-gallocatechin gallate and myricetin rhamnoside within the active pockets of 5-LOX, COX-1 and COX-2 were illustrated in Figure 9.

However, the effect of ionization of different functional groups as well as amino acid moieties at the active site is well interpreted by the rule-based ionization method. This was reflected by the formation of an extra ionic bond between the phenolic hydroxyl groups that dissociate to form negatively charged phenolate ions in (epi)-gallocatechin gallate and positively charged amino groups as Arg 106 at COX-1 active site. Furthermore, an ionic bond was also observed between Arg 106 at COX-2 active site and the negatively charged phenolate ions in myricetin rhamnoside.

**DISCUSSION**

The present study profiled the phytoactive secondary metabolites and investigated the antioxidant, hepatoprotective, anti-inflammatory and analgesic activities of *S. aqueum* leaf extract in animal models and the underlying mechanisms. Utilizing LC-MS/MS, 87 compounds were tentatively identified, belonging to flavonoids, ellagitannins and proanthocyanidins. Promising antioxidant activities were obtained in all *in vitro* assays and in a cell-based model against the deleterious effects of UVA radiations. Also, the extract was able to counteract the deleterious effects of CCl₄ in rats. These results are similar to previous findings from *Syzygium samarangense*, *Syzygium jambos* leaves and from other *Syzygium* species (Sobeh et al., 2018a,b).

We also assessed the anti-inflammatory activity of the extract *in vitro* by carrageenan-induced peritonitis, a suitable model of acute inflammation, in which vascular changes and production of inflammatory cytokines leading to leukocyte migration. We focused on the early increase in vascular permeability and neutrophil infiltration into peritoneum. The extract inhibited both cyclooxygenases, COX-1 and COX-2 with more inhibitory potential toward COX-2. Its effect on COX-2 was more potent than diclofenac but less than that of celecoxib. Furthermore, the extract inhibited lipoxygenase (LOX), thus suppressed leukotriene synthesis and inhibited RBC hemolysis.

In addition, the extract attenuated the carrageenan induced rat hind paw edema and inhibited carrageenan induced leukocyte migration. The effect of the extract was comparable but less potent than diclofenac. Also, the leaf extract demonstrated anti-inflammatory activity and produced comparable inhibition of leukocyte migration in comparison with diclofenac. The response to pain stimuli in the hot plate test is attributed to the supraspinal reflex mediated by \(\mu\)-opioid receptors (Le Bars et al., 2001). The antinociceptive activity is characterized by an increased tolerance to pain by the animal in contact with a heated plate. In the present study, animals pre-treated with the extract (300 mg/kg, p.o) significantly prolonged the response latency when measured at 1 and 2 h after administration. These results indicate that the extract also possessed central antinociceptive activity.

Altogether, the extract possesses solid antioxidant, hepatoprotective, anti-inflammatory, and pain-killing activities *in vivo*. These effects may be attributed to its flavonoid content, especially myricetin rhamnoside, quercetin glucosides, and epigallocatechin gallate. Flavonoids, among them quercetin, and tannins possess antioxidant, hepatoprotective and anti-inflammatory effects and play an important role in alleviating acute inflammation (Singh and Pandey, 1997). The anti-inflammatory effect of some flavonoids may be due to the significant inhibitory potential against many enzymes involved in inflammation such as phosphodiesterase, phospholipase A2, protein tyrosine kinases, and others. Furthermore, many flavonoids inhibit the major enzymes involved in the synthesis of prostaglandins and leukotrienes such as COX-1, COX-2 and LOX (Manthey et al., 2001). The extract is rich in polyphenols, which carry several reactive phenolic hydroxyl groups; they can partly dissociate to negatively charged phenolate ions. Polyphenols can form several hydrogen and ionic bonds with most proteins. As a consequence proteins complexed with polyphenols change their 3D structures and thus modulate their bioactivities (Wink, 2015; van Wyk and Wink, 2017). As this polyphenol-protein interaction is not specific, several proteins are involved, which would also explain the pleiotropic activities of our extract.

The results observed in the different *in vitro* and *in vivo* assays showed that *S. aqueum* offers good protection against oxidative stress, free radicals and oxidative stress related diseases. More detailed pharmacological assays are required before the findings can be translated into applications in humans.

**ETHICS STATEMENT**

Hepatoprotective activities: The study protocol was approved by the Unit of Biomedical Ethics Research Committee, Faculty of Medicine, King Abdulaziz University, following the Institutional Animal Care and Use Committee guidelines. Anti-inflammatory and antinociceptive activities: The animal experiments were approved by the Ethical Committee of the Faculty of Pharmacy, Zagazig University for Animal Use and conducted following the guidelines of the US National Institutes of Health on animal care and use. Approved protocol for animal treatment is: P 9-12-2017, Faculty of Pharmacy, Zagazig University, Egypt.

**AUTHOR CONTRIBUTIONS**

MS performed the extraction, chemical characterization of the extract, the antioxidant activities *in vitro*, analyzed the data, wrote the paper, and conceived and designed the project. MM and SR designed and performed the anti-inflammatory and analgesic...
experiments and wrote the paper. GP and DM performed the antioxidant activities in keratinocytes and wrote this part. MA and FY performed the molecular modeling experiments and wrote this part. AF-S analyzed the data and revised the paper. AA-N performed the hepatoprotective activities and MW revised the paper, conceived and designed the project.

**FUNDING**

The authors received financial support from the Deutsche Forschungsgemeinschaft and Ruprecht-Karls-Universität Heidelberg within the funding program Open Access Publishing.

**REFERENCES**

Abdelall, E. K. A., Lamie, P. F., and Ali, W. A. M. (2016). Cyclooxygenase-2 and lipoxigenase inhibition, synthesis, anti-inflammatory activity and ulcer liability of new celecoxib analogues: determination of region-specific pyrazole ring formation by NOESY. Bioorg. Med. Chem. Lett. 26, 2893–2899. doi: 10.1016/j.bmcl.2016.04.046

Ashour, M. L., Youssef, F. S., Gad, H. A., and Wink, M. (2017). Inhibition of cytochrome P450 (CYP3A4) activity by extracts from 57 plants used in traditional chinese medicine (TCM). Pharmaceut. Mag. 13, 300–308. doi: 10.4103/0973-1296.204561

Eddy, N. B., and Leimbach, D. (1953). Synthetic analogues. II. Dithiénylbutenyl- and dithiénylbutyramines. J. Pharmacol. Exp. Ther. 107, 385–393.

Finkel, T., and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247. doi: 10.1038/35041687

Ghareeb, M. A., Mohamed, T., Saad, A. M., Refahy, L. A.-G., Sobeh, M., and Wink, M. (2017). HPLC-DAD-ESI-MS/MS analysis of fruits from *Firmiana simplex* (L.) and evaluation of their antioxidant and antigenotoxic properties. J. Pharm. Pharmacol. 70, 133–142. 10.1111/jphp.12843.

Le Bars, D., Gozariu, M., and Cadden, S. W. (2001). Animal models of nociception. Pharmacol. Rev. 53, 597–652.

Macdonald, A. D., Woolfe, G Bergel, F., Morrison, A. L., and Rinderknecht, H. (1946). Analytical action of pethidine derivatives and related compounds. Br. J. Pharmacol. Chemother. 1, 4–14. doi: 10.1111/j.1476-5381.1946.tb00222.x

Manaharan, T., Appleton, D., Cheng, H. M., and Palansamy, U. D. (2012). Flavonoids isolated from *Syzygium aqueum* leaf extract as potential antihyperglycaemic agents. Food Chem. 132, 1802–1807. doi: 10.1016/j.foodchem.2011.11.147

Manthey, J. A., Guthrie, N., and Grohmann, K. (2001). Biological properties of citrus flavonoids pertaining to cancer and inflammation. Curr. Med. Chem. 8, 135–153.

Monti, D. M., Guarnieri, D., Napolitano, G., Piccoli, R., Netti, P., Pedrono, R. B., Spironello, R. A., et al. (2014). Effect of estragole on leukocyte behavior and phagocytic activity of macrophages. Evid. Based Complement. Alternat. Med. 2014:784689. doi: 10.1155/2014/784689

Singh, R., and Pandey, B. (1997). Further study of antiinflammatory effects of Abies pindrow. Phytother. Res. 11, 535–537. doi: 10.1002/(SICI)1099-1573(199711)11:7<535::AID-PTR146>3.0.CO;2-7

Sobeh, M., Mamadaliева, N. Z., Mohamed, T., Krstин, S., Youssef, F. S., Ashour, M. L., et al. (2016). Chemical profiling of *Phloemis thapsoides* (Lamiaceae) and in vitro testing of its biological activities. Med. Chem. Res. 25, 2304–2315. doi: 10.1007/s00444-016-1677-9

Sobeh, M., Esmat, A., Petrug, G., Abdelfattah, M. A. O., Dmitriev, M., Monti, D. M., et al. (2018a). Phenolic compounds from *Syzygium jambos* (Myrtaceae) exhibit distinct antioxidant and hepatoprotective activities. (in *ijo*. J. Funct. Foods 41, 223–231.

Sobeh, M., Youssef, F. S., Esmat, A., Petrug, G., El-Khatib, A. H., Monti, D. M., et al. (2018b). High resolution UPLC-MS/MS profiling of polyphenolics in the methanol extract of *Syzygium samarangense* leaves and its hepatoprotective activity in rats with CCl4-induced hepatic damage. Food Chem. Toxicol. 113, 145–153. doi: 10.1017/0929867013373723

van Wyk, B.-E., and Wink, M. (2015). Phytoedicines, Herbal Drugs, and Poisons. Chicago, IL: University of Chicago Press.

van Wyk, B.-E., and Wink, M. (2017). *Medicinal Plants of the World*, 2nd Edn. Portland: Timber Press Inc.

Wink, M. (2015). Modes of action of herbal medicines and plant secondary metabolites. Medicines 2, 251–286. doi: 10.3390/medicines2030251

Winter, C. A., Risley, E. A., and Nuss, G. W. (1962). Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. Proc. Soc. Exp. Biol. Med. 111, 544–547. doi: 10.3181/00379727-111-27849

Youssef, F. S., Ashour, M. L., Ebada, S. S., Sobeh, M., El-Beshbishy, H. A., Singab, A. N., et al. (2017). Antihyperglycaemic activity of the methanol extract from leaves of *Eremopha ma cristula* (Scrophulariaceae) in streptozoicin-induced diabetic rats. J. Pharm. Pharmacol. 69, 733–742.

Zhang, G. Q., Huang, X. D., Wang, H., Leung, A. K., Chan, C. L., Fong, D. W., et al. (2008). Anti-inflammatory and analgesic effects of the ethanol extract of *Rosa multiflora* Thunb. hps. J. Ethnopharmacol. 118, 290–294.

**ACKNOWLEDGMENTS**

The authors would like to thank Dr. A. H. El-khatib, Department of Chemistry, Humboldt-Universität zu Berlin, Berlin, Germany, for his help in collecting the LC-MS data.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00566/full#supplementary-material

Sylva-Comar, F. M., Wizirler, L. A., Silva-Filho, S. E., Kummer, R., Pedrono, R. B., Spironello, R. A., et al. (2014). Inhibition of cytochrome P450 (CYP3A4) activity by extracts from 57 plants used in traditional chinese medicine (TCM). Pharmacogn. Mag. 13, 300–308. doi: 10.4103/0973-1296.204561

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SC and handling Editor declared their shared affiliation.

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