Antioxidant, Analgesic, Antimicrobial and Molecular Docking Studies of the Leaves of *Oroxylum indicum* (L.) Kurz.

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ABSTRACT: This study presents the findings of *in vivo*, *in vitro* and *in silico* investigations of the *Oroxylum indicum* (L.) Kurz leaf extracts. Various fractions namely chloroform (CF), ethyl acetate (EAF), *n*-hexane (HF) and aqueous fractions (AF) obtained from the crude extract were subjected to biological investigations. In DPPH based free radical scavenging assay, CF and MF fractions showed potent antioxidant activity with IC_{50} 9.70 µg/ml and 10.46 µg/ml as compared to IC_{50} 8.56µg/ml for the standard ascorbic acid. The CF and HF fractions of *O. indicum* (L.) Kurz leaves exhibited mild antimicrobial activity. In analgesic activity screening by acetic acid induced writhing method, EAF showed significant writhing inhibition of 68% and 72%, at doses 150 mg/kg and 300 mg/kg, respectively. In tail immersion method, EAF also showed potent analgesic activity with reaction time of 1.816 ± 0.047 and 2.15 ± 0.041 min, after 30 minutes following oral doses of 150 mg/kg and 300 mg/kg, respectively. Molecular docking of previously isolated compounds from *O. indicum* (L.) Kurz leaf extracts were docked against cyclooxygenase-1 (COX-1) and cyclooxygenase -2 (COX-2) and revealed that the flavonoids baicalein and chrysin may be responsible for the analgesic activity. Lipinski's rule of five was also calculated for the compounds to assess drug likeliness.

Key words: *Oroxylum indicum* (L.), anti-oxidant, analgesic, molecular docking simulation

INTRODUCTION

For thousands of years plants have been used for therapeutic purposes owing to their medicinal values. Plants are a great source of structurally complex bioactive compounds. The ethnopharmacological properties of the plants have been used as a potential source of medicines and lead compounds for drug discovery.[1,2] Plants utilized in traditional medicine and treatment methods from many civilizations around the world are thus fascinating research subjects.

O. indicum (L.) Kurz has been used for centuries as a traditional medicine in Asia such as in Ayurvedic system of medicine as single drug or in combination with other compounds.[3] It is a species of flowering plant belonging to the monotypic genus *Oroxylum* in the family Bignoniaceae.[4] It is commonly known as Sonapatha and is native to the Indian subcontinent, the foothills of the Himalayas spreading to the regions of Bhutan and southern China, Malaysia and Indochina.[5] In Bangladesh, this plant is usually found in the forests of Chittagong Hill Tracts, Cox`s Bazar and Tangail.[6] Various parts of *O. indicum* (L.) Kurz including leaves, stem bark, root bark, fruits, seeds and heartwood, contain diverse phytochemicals, such as chrysin, baicalein, oroxylin-A, oroxindin, emodin and other anthraquinones, prunetin, sitosterol, ellagic acid and tetuin etc.[3-5]

Extracts obtained from different parts of *O. indicum* (L.) Kurz have been investigated and found to exhibit manifold pharmacological activities. Extracts from these parts have been documented to possess anti-inflammatory,[7] antimicrobial,[8,9] antioxidant,[10,11] anticancer,[12,13] antiarthritic[14] hepatoprotective[15] and cardioprotective[16] activities. Although root, stem and stem-bark of this plant have been investigated extensively, leaf extracts of this

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plant have been examined only few instances. These include antiproliferative effect of methanol extract,[17] anti-inflammatory effect of aqueous extract,[18] glucose lowering effect of aqueous and ethanol extract[19] etc. In this study, various fractions such as methanolic, *n*-hexane, ethyl acetate, chloroform and aqueous fractions of leaves were subjected to several biological investigations, such as assessment of antioxidant, antimicrobial and analgesic activity, many of which have not been reported previously. In addition to the above, in *silico* studies such as molecular docking and drug likeliness property calculation of the phytoconstituents of the active fractions have been accomplished to complement biological studies and to gain new insights about pharmacological effect of different extracts.

MATERIALS AND METHODS

Collection and identification of the plant. Plant sample of *O. indicum* (L.) Kurz was collected in September-2019 from Sylhet district and the authenticity of the plant was confirmed by the assigned specialist from Bangladesh National Herbarium (BNH).The voucher specimen was also deposited (DACB accession number 57244).

Preparation of plant samples. Leaves of the plant were cleaned properly to remove dirt and other impurities. Adequately cleaned leaves went under shade drying for a week. The leaves were then oven dried for a duration of 24 hours at 37°C to facilitate grinding process. The dried leaves were then crushed to form coarse powder state by a grinding machine of high capacity. Subsequently, the powder was stored in a suitable airtight container with identification label. Finally, the container was preserved in a dark place with controlled temperature and humidity for the investigations.

Solvents and reagents. All the solvents and reagents used during this investigation were analytical or laboratory grade which were procured from either E. Merck (Germany) or BDH (England). The organic solvents utilized in extraction process such as methanol, *n*-hexane, ethyl acetate and chloroform were distilled and well dried before use.

Extraction of the plant material. *O. indicum* (L.) Kurz extract was formulated by cold extraction process.[20] Around 1500 gm of powdered material of the plant was immersed in 3 liters of distilled methanol in proper container The container was kept in place for 30 days where it was shaken and stirred from time to time. The resultant mixture was filtered through a fresh and clean cotton plug and subsequently with a Whatman No.1 filter paper. The filtrates were then concentrated using a vacuum rotary evaporator. Solvent-solvent partitioning was accomplished following the protocol devised by Kupchan and modified by Wagenen.[21] The crude extract weighed 5 gm. Later it was dissolved in 10% aqueous methanol. It was extracted with n-hexane, then with chloroform and finally with ethyl acetate.

Experimental animal. Swiss-albino mice (*Mus musculus*) collected from Jahangir Nagar University Animal House, Savar, Bangladesh were used in the evaluation of analgesic activity. The animals were of either sex and aged between 4-5 weeks. Ideal polypropylene cages (30cm x 20cm x 13cm) were used to keep the animals in standard conditions (temperature 21 ± 1° C with a 12 h light and dark cycle) for seven days prior to the analgesic experiments. The animals were fed approved standard diet and water as required.

Pharmacological screening. In this research work the free radical scavenging, antimicrobial and analgesic activities of different solvent fractions of crude methanolic extract of *O. indicum* (L.) Kurz were investigated.

Free radical (DPPH) scavenging activity. The antioxidant potential was evaluated by calculating percent inhibition of DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical.[22] Ascorbic acid was used as standard in the assay. Free radical inhibition percent (I %) was calculated by using the following equation:

$$I\% = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}}\right) \times 100\%$$

Concentration of the extracts that provides 50% inhibition (IC_{50}) of the free radical DPPH was

derived from the logarithmic curve of percentage inhibition versus concentration of the sample.

Antimicrobial activity. Antimicrobial activity was investigated by disc diffusion method.[23]. Sterilized discs of 6 mm diameter were used and these were diffused with 400 μg/disc of extracts. After that, discs were transferred to petri-dishes for culturing with microbes. After incubation, the antimicrobial activities of the extracts were estimated by measuring the diameter of the zones of inhibition expressed in millimeter. The bacterial species under investigation contained both Gram (+) and Gram (-) species. A total of 16 bacterial species and 3 fungal species were used in the experiment.

Analgesic activity. Analgesic activities of the extracts were evaluated by two methods. Acetic acid induced writhing method was employed to evaluate peripheral analgesic effect and tail immersion method to assess central analgesic effect.[24,25] In peripheral analgesic assay, sixty mice were allotted into twelve different equal groups arbitrarily . Negative control group was supplied with 0.9% NaCl, positive control group with diclofenac (25 mg/kg) and other groups received plant extracts (150 and 300 mg/kg) by oral administration. Forty minutes later, writhing was induced by administration of acetic acid (0.7% v/v, 10 ml/kg) intra-peritoneally to all of the groups. Writhing count started after five minutes and was counted for the next fifteen minutes for each mouse.

In central analgesic assay, activities of the plant extracts were investigated by tail immersion method with a hot water bath.[25] Temperature of the bath was maintained between 55 ± 0.5 °C. Sixty mice were allocated to twelve equal groups. Positive control group received morphine (5 mg/kg), negative control group received 0.9% NaCl and other groups were given plant extracts (150 and 300 mg/kg). The tail flicking time was noted at time intervals of 0, 30, 60 and 90 min.

Statistical analysis. Values were presented as the mean ± standard error of mean (SEM). The one-way analysis of variance (ANOVA) and Dunnett's test were performed where $P<0.05$ was regarded as statistically significant.

Molecular docking analysis and Lipinski's rule of five prediction. The structures of proteins cyclooxygenase-1 (COX-1) (PDB code: 1EQG, resolution: 2.61 Å), cyclooxygenase-2 (COX-2) (PDB code: 5IKT, resolution: 2.45 Å) were taken from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB).[26] Ligands and co-crystallized water molecules were removed from the protein molecules and polar hydrogens were added using Discovery Studio Client 2019. Protein minimization was accomplished at YASARA Energy Minimization Server (http://www.yasara.org/ minimizationserver.htm) using YASARA force field level of theory. Open Babel (version 2.4.0) was used for energy minimization of the compounds used in docking.[27] Docking protocol validation procedure was executed by re-docking the respective native ligands in the binding sites of the proteins.[28] The target protein-ligand docking was accomplished using AutoDock Vina (version 1.1.2).[29] In docking simulation, centers of the grids were located at the active site of respective proteins. The grid coordinates (X, Y, Z) for COX-1 and COX-2 were (27.48, 38.48, 200.04) and (164.39, 183.38, 195.80), respectively. The dimensions of the grids used were 25.00, 25.00, 25.00 Å. The possible interactions of ligands with proteins were investigated and 2D figures were obtained by Discovery Studio Client 2019.

Parameters that predict the likelihood of compounds to be drug like (Lipinski's rule of five) i.e. molecular weight, hydrogen bond donors and acceptors present in the structures, lipophilicity, molar refractivity of the selected compounds were estimated using SwissADME.[30]

RESULTS AND DISCUSSION

Antioxidant activity. Anti-oxidant potential of different fractions of *O. indicum* (L.) Kurz leaf extract was estimated exploiting the ability of different fractions in quenching DPPH free radical. In our investigation, different fractions from leaf extract showed anti-oxidant potential in the following pattern: CF > MF > HF > EAF > AF. CF fraction

showed the highest free radical scavenging activity followed by methanol fraction, with IC_{50} value 9.07 µg/mL and 10.46 µg/ml, respectively which are comparable to that of standard ascorbic acid (IC_{50} value of 8.56 µg/ml). Samatha *et al.* (2012) reported the flavonoid content of CF and MF extract were 1.44 and 1.12 catechin equivalent per gram of dry weight.[31] Hence, the anti-oxidant potential of the CF and MF extract may be due to the phytochemical diversity of phenolic content of the extracts. Other fractions did not exhibit potential activity compared to the standard. Figure 1 shows the comparative IC_{50} values of the standard and different fractions.

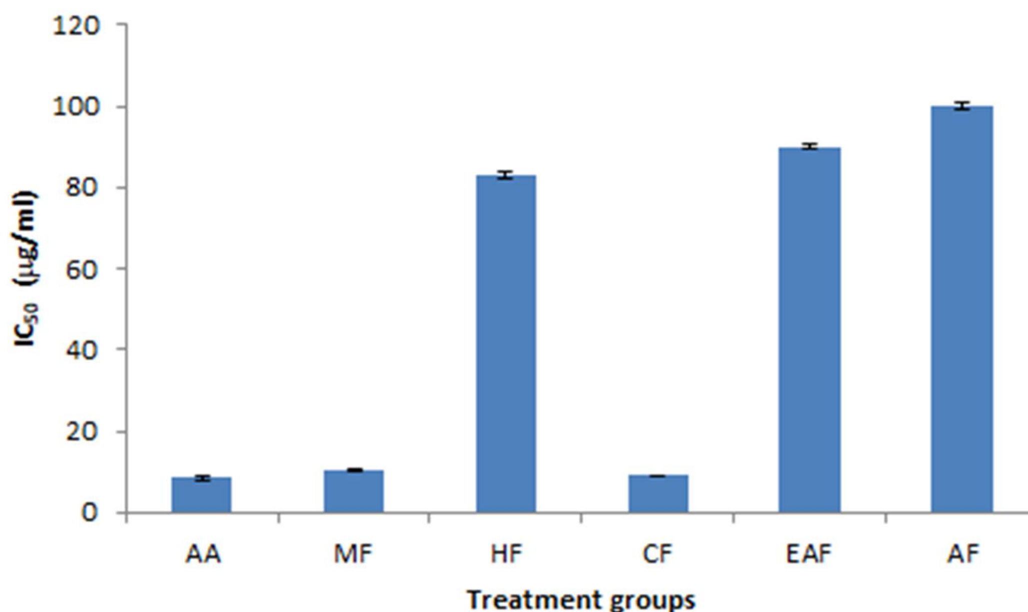

Figure 1: IC_{50} values of the standard and different fractions *Oroxylum indicum* (L.) Kurz leaves. AA: ascorbic acid, MF: methanolic fraction, HF: *n*-hexane soluble fraction, CF: chloroform soluble fraction, EAF: ethyl acetate soluble fraction, AF: aqueous fraction

Oxidative stress which is characterized by over prevalence of free radical in body than antioxidant is thought be cause for many health complications.[32] Plant polyphenols have shown potential antioxidant activities and therefore these secondary plant metabolites might be the interesting candidates for developing new anti-oxidant supplements.[33] Since, the CF and MF fractions *O. indicum* (L.) Kurz leaf extract showed potential activity further compound specific investigation of anti-oxidant activity can lead to development of new anti-oxidant supplement.

Antimicrobial activity. Anti-microbial effect of bacterial species and fungal species were compared to ciprofloxacin (10 µg/disc) and griseofulvin disc (10 µg/disc), respectively. MF, HF and CF fractions of *O. indicum* (L.) Kurz showed mild to negligible antibacterial and antifungal activity in several species. Table 1 summarizes the antimicrobial effect of standards and different fractions

Analgesic activity. The effects of the plant extracts in reducing the nociception caused by acetic acid in terms of decreasing writhing are presented in Table 2. In this method EAF fraction showed promising analgesic activity with 68% and 72% inhibition of writhing at doses of 150 mg/kg and 300 mg/kg, respectively. Diclofenac inhibited writhing by 80% at a dose of 25 mg/kg. At 300 mg/kg dose AF, CF and HF fractions showed writhing inhibition of 68%, 64% and 60%, respectively.

Table 1. Antimicrobial activity of different fractions of *O. indicum* (L.) Kurz leaves by measuring the zone of inhibition.

Test bacteria and fungi	Ciprofloxacin/ griseofulvin	MF	HF	CF	EAF	AF
Bacillius cereus	50	10	9	10	8	0
Bacillius megaterium	48	0	0	0	0	0
Bacillius subtilis	48	9	10	9	8	0
Salmonella paratyphi	47	8	8	8	0	9
Salmonella typhi	47	7	8	9	0	0
Vibrio parahemolyticus	48	8	8	7	0	7
Vibrio mimicus	49	9	8	7	0	0
Staphylococcus aureus	50	9	9	8	0	0
E. coli	48	9	7	8	0	0
Shigella dysenteriae	50	7	9	8	0	0
Pseudomonas aureus	48	8	8	8	0	0
Sarcina lutea	49	7	9	9	0	0
Shigella boydii	47	8	9	9	0	0
Saccharromyces cerevisiae	46	9	8	8	0	0
Candida albicans	46	0	0	0	0	0
Aspergillus niger	46	9	9	8	0	0

Zone of inhibition is measured in mm. MF: methanolic fraction, HF: *n*-hexane soluble fraction, CF: chloroform soluble fraction, EAF: ethyl acetate soluble fraction, AF: aqueous fraction

Table 2. Analgesic activity of plant extracts in acetic acid induced writhing method.

Treatment	Dose (mg/kg)	Average writhing count	Inhibition of writhing (%)
Negative Control	0.1 ml/10gm	25 ± 0.632	0
Diclofenac	25 mg/kg	5 ± 0.632***	80
MF	150	15 ± 1.523***	40
MF	300	12 ± 1.414***	52
HF	150	11 ± 1.058***	56
HF	300	10 ± 0.632***	60
CF	150	10 ± 0.632***	60
CF	300	9 ± 0.632***	64
EAF	150	8 ± 0.632***	68
EAF	300	7 ± 0.632***	72
AF	150	10 ± 0.632***	60
AF	300	8 ± 0.632***	68

Writhing counts are represented as mean ± SEM,(n=5). Groups treated with different extracts and diclofenac are compared with negative control. MF: methanolic fraction, HF: *n*-hexane soluble fraction, CF: chloroform soluble fraction, EAF: ethyl acetate soluble fraction, AF: aqueous fraction ***P<0.001meaning significant differences.

Central analgesic activity of the plant extracts were compared by tail flick method and the data are presented in Table 3. EAF fraction at a dose 300 mg/kg showed most potent tail flick reaction time of 2.15 ± 0.041 min among the plant extracts which is comparable to reaction time of 3.316 ± 0.069 min of morphine after 30 min of oral administration. EAF fraction at both doses showed superior reaction time than other fractions at compared at all the time intervals. Other fractions exhibited mild to moderate analgesic activity.

In acetic acid induced writhing method, administration of acetic acid through intra peritoneal route initiates an acute inflammatory response followed by activation of the nociceptors.[34] The tail flick method is a widely accepted method to assess anti-nociceptive property of plant extracts. Pain sensitive receptors at the tail of mice, transient receptor potential vanilloid type 1 and 3 (TRPV1 and TRPV3) receptors are activated when tail is exposed to heat inducing pain transmission.[35] EAF fraction exerted potent analgesic activity in both the tests. MF and HF fractions also showed moderate analgesic activity. The analgesic activity of leaf extract may be due to phytochemical constituents present in the extracts possibly flavonoids because flavonoids are reported to possess analgesic activity.[36] Further *in vivo and in silico* studies might give more idea about the mechanism of pain inhibition property of the extracts.

Table 3. Evaluation of central analgesic effect of extracts using tail flick method in mice.

Treatment	Tail Flicking Time in minutes			
	0 minute	30 minute	60 minute	90 minute
Negative control	0.766 ± 0.041	0.788 ± 0.034	0.744 ± 0.032	0.674 ± 0.034
Morphine(5mg/kg)	1.578 ± 0.081***	3.316 ± 0.069***	2.86 ± 0.054***	3.054 ± 0.048***
MF (150 mg/kg)	0.884 ± 0.051*	1.77 ± 0.042***	0.72 ± 0.037	1.67 ± 0.028***
MF (300 mg/kg)	0.996 ± 0.009***	1.416 ± 0.138**	1.0002 ± 0.006***	1.65 ± 0.082***
HF (150 mg/kg)	0.794 ± 0.032	0.854 ± 0.035	0.086 ± 0.034	0.766 ± 0.042
HF (300 mg/kg)	1.082 ± 0.038***	1.372 ± 0.078***	1.026 ± 0.023***	0.98 ± 0.029***
CF (150 mg/kg)	0.861 ± 0.060	0.652 ± 0.031*	0.666 ± 0.048	0.764 ± 0.031
CF (300 mg/kg)	1.158 ± 0.036***	1.080 ± 0.032***	1.1034 ± 0.025***	0.954 ± 0.029***
EAF (150 mg/kg)	0.837 ± 0.036	1.816 ± 0.047***	1.604 ± 0.046***	0.934 ± 0.048**
EAF (300 mg/kg)	1.233 ± 0.059***	2.150 ± 0.041***	1.928 ± 0.025***	1.86 ± 0.089***
AF (150 mg/kg)	0.774 ± 0.030	0.752 ± 0.038	0.762 ± 0.036	0.546 ± 0.030**
AF (300 mg/kg)	0.733 ± 0.039	1.001 ± 0.006***	0.686 ± 0.031	0.848 ± 0.035**

Reaction time values are expressed as mean ± SEM, (n = 5). Groups treated with different extracts and morphine are compared with negative control. MF: methanolic fraction, HF: *n*-hexane soluble fraction, CF: chloroform soluble fraction, EAF: ethyl acetate soluble fraction, AF: aqueous fraction ***P < 0.001, **P < 0.01, *P < 0.05 meaning significant differences.

Molecular Docking and Lipinski's rule of five prediction. To investigate the reason for analgesic activity of the leaf extract particularly the active fraction EAF, molecular docking of compounds reported from EAF fraction of *O. indicum* (L.) Kurz leaves were performed against the isoenzymes Cyclooxygenase 1 (COX-1) and Cyclooxygenase 2 (COX-2). COX isoenzymes are important because they are the pharmacological targets of non-steroidal anti-inflammatory drugs which are used to subside pain and reduce inflammation.[37] Two flavonoids such as baicalein and chrysin and their glycosides have been reported to be isolated from EAF.[38] These compounds along with standard diclofenac have been docked to the active site of COX enzymes. Structures of diclofenac and the plant compounds docked are shown in Figure 2.

The binding affinities obtained from molecular docking and the values of important physicochemical parameters important for drug development have been enlisted in Table 4. And the binding interactions of the compounds with COX-1 and COX-2 are shown in Figure 3 and Figure 4, respectively. Baicalein showed equal binding affinity of -8.3 kcal/mol like diclofenac with COX-1 active site. Both baicalein formed hydrogen bond with Ser530 residue of COX-1 similar to diclofenac. Ser530 residue is very crucial for initiation of prostaglandin synthesis and is acetylated by aspirin exerting analgesic effect.[39] Chrysin, another important flavonoid from EAF fraction, showed comparable

binding affinity of -8.2 kcal/mol with COX-1 active site forming hydrogen bonding with Ser530 and Met522 residue of COX-1.

Chrysin showed preferential binding with COX-2 than COX-1 with a binding affinity of -8.7 kcal/mol which is also higher than the binding affinity of diclofenac (-8.4 kcal/mol) forming hydrogen bonding with Ser530 residue. However, baicalein showed less affinity for COX-2 than COX-1. Oroxin A and Oroxin B which are baicalein 7-*O*-glucoside and Baicalin-7-diglucoside respectively, showed lesser binding affinity than their aglycone part baicalein both with COX-1 and COX-2. Chrysin 7-*O*-glucoronide showed considerable binding affinity of -8.0kcal/mol with COX-2 forming hydrogen bonding with Gln 350 and Tyr 355 residue of COX 2 active site. Efficient binding of the flavonoids baicalein and chrysin can partly explain why EAF fraction showed potent analgesic activity. However, further compound specific *in vivo*, *in vitro* and *in silico* study can establish the exact mechanism of analgesic activity more clearly.

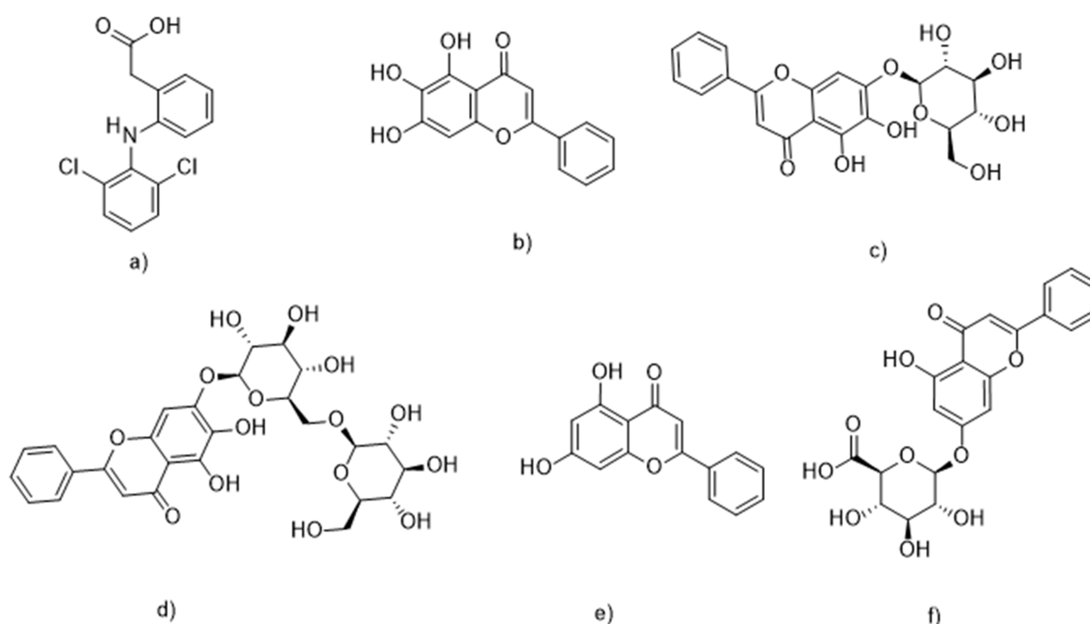

Figure 2. Structure of compounds used in molecular docking simulation. a) Diclofenac (PubChem CID 3033) b) Baicalein (PubChem CID 5281605) (c) Oroxin A (PubChem CID 5320313) d) Oroxin B (PubChem CID 10077207) e) Chrysin (PubChem CID 5281607) f) Chrysin 7-O –glucoronide (PubChem CID 14135335)

Table 4. Binding affinities of the docked plant compounds and Lipinski's rule of five prediction compared to standard diclofenac.

Compounds	Binding affinity (kcal/mol)		Drug like property prediction (Lipinski's rule of five)				
	COX-1	COX-2	Molecular weight	H-bond acceptor	H-bond donor	Molar Refractivity	LogP
Diclofenac	-8.3	-8.4	296.15	2	2	77.55	3.84
Baicalein	-8.3	-7.8	270.24	5	3	73.99	0.52
Oroxin A	-7.7	-7.1	432.38	10	6	106.11	-1.61
Oroxin B	-7.2	-5.1	594.52	15	9	138.49	-3.69
Chrysin	-8.2	-8.7	254.24	4	2	71.97	1.08
Chrysin 7-O -glucoronide	-7.5	-8	430.36	10	5	104.70	-1.14

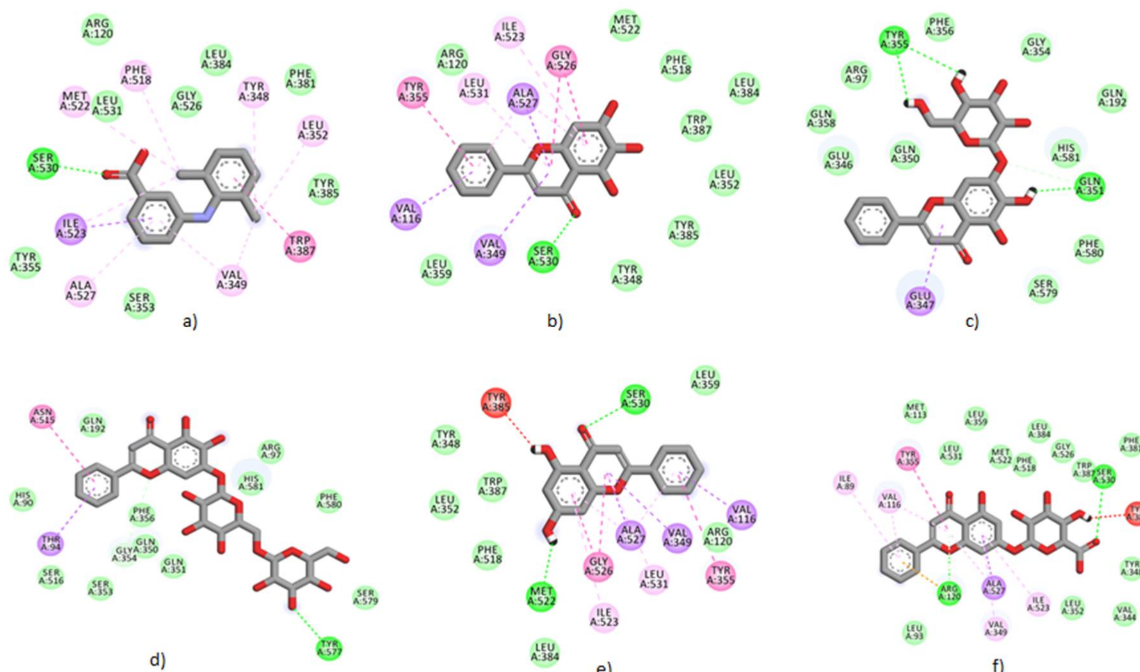

Figure 3. Binding interaction of a) dicofenac, b) baicalein, c) oroxin A, d) oroxin B, e) chrysin and f) chrysin 7-O –glucoronide with COX 1 (1EQG). Green: conventional hydrogen bond; cyan: carbon hydrogen; violet-pink: hydrophobic; light green: Van der Waals; orange: pi-anion/pi-cation; red: donor-donor interaction.

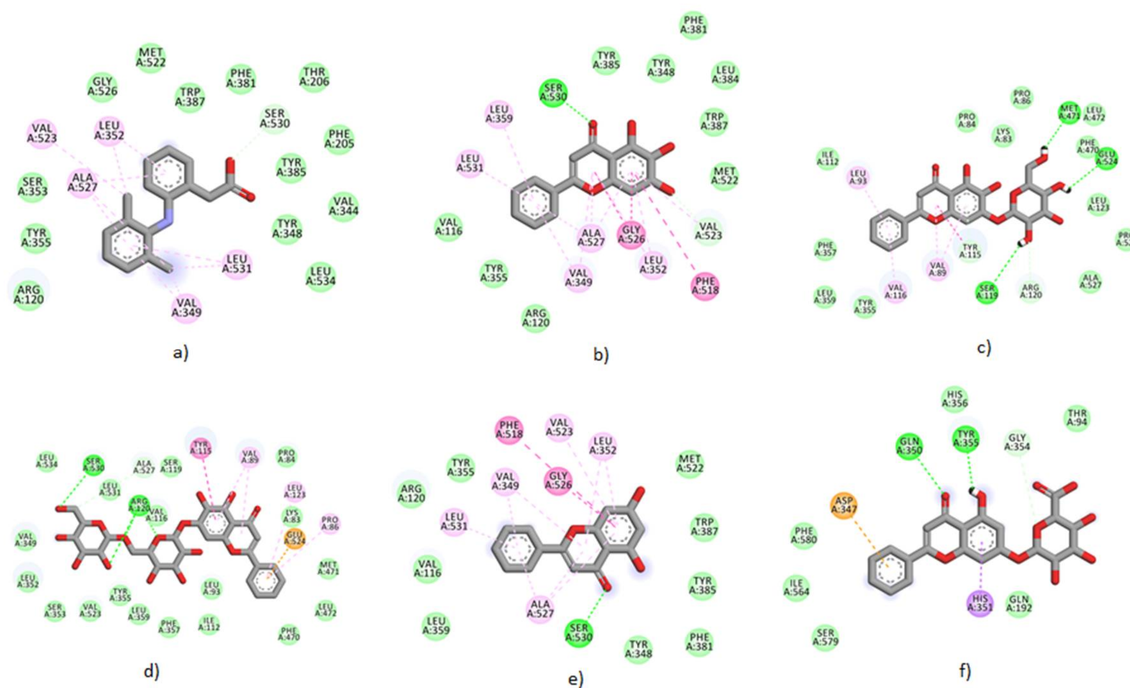

Figure 4. Binding interaction of a) dicofenac, b) baicalein, c) oroxin A, d) oroxin B, e) chrysin and f) chrysin 7-O –glucoronide with COX 2 (5IKT). Green: conventional hydrogen bond; cyan: carbon hydrogen; violet-pink: hydrophobic; light green: Van der Waals; orange: pi-anion/pi-cation; red: donor-donor interaction.

Lipinski's rule of five describes physicochemical parameters important predictive for good pharmacokinetic properties during drug development. The acceptable criteria are: molecular weight <500, number of hydrogen bond donor ≤ 5, number of hydrogen bond acceptor ≤ 10, molar refractivity between 40-130 and octanol-water partition coefficient (LogP) <5.[40] Baicalein, chrysin and chrysin 7-*O*–glucoronide fulfilled all the criteria meaning these compounds may have good pharmacokinetic outcomes while drug development. Oroxin A and Oroxin B which showed lower binding affinity in molecular docking also violated one (number of hydrogen bond donor) and three (molecular weight, number of hydrogen bond acceptor, number of hydrogen bond donor) parameters of Lipinski's rule of five.

CONCLUSION

Different fractions of *O. indicum* (L.) Kurz leaf extract were subjected to free radical scavenging activity, antimicrobial and analgesic assay. Chloroform and methanol fractions demonstrated antioxidant activity. Methanol, chloroform and *n*-hexane fractions exhibited very mild antibacterial and antifungal activity. Ethyl acetate fraction exhibited potent analgesic activity in both peripheral and central analgesic assay. Molecular docking of compounds from ethyl acetate fraction revealed that the flavonoids baicalein and chrysin may be responsible for analgesic activity of the fraction. Therefore, given the potential bioactivity, plant extract and its constituents can be further extensively studied to assess their unexplored effectiveness and to rationalize their uses as traditional medicines and new drug development.

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