Purification and Analysis of Salicinoids

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Abstract: Background: Salicinoids (a type of phenolic glycoside) are plant secondary metabolites with chemical structures based on salicyl alcohol conjugated to \(\beta\)-D-glucopyranose, with demonstrated anti-herbivore activity. These compounds have been purified and quantified in a variety of contexts. Validation of published methods is often incomplete, and there is no broadly-applicable reference procedure.

Objective: To develop and validate a robust, versatile procedure for purification and quantification of salicinoids in salicaceous plants.

Method: We extracted salicinoids from dried, ground \textit{Populus} foliage into methanol:water, and purified them by sequential liquid-liquid extraction, flash chromatography and preparative scale HPLC. To evaluate potential source material for purification of salicinoids, we quantified salicortin, hydroxyceclohexen-on-oyl salicortin (HCH-salicortin), and tremulacin in methanolic extracts of \textit{Populus tremuloides}, \textit{P. fremontii}, and \textit{P. deltoides} using ultra-high performance liquid chromatography (UHPLC) with diode array (DAD) and negative electrospray ionization single quadrupole mass spectrometry (MS) detection.

Results: Recovery efficiencies and purities of salicinoids extracted from \textit{Populus} ranged from 6-63\% and 58->99\%, respectively. Both detectors provided accurate quantification of salicinoids; MS was 100\times more sensitive than DAD, permitting detection of plant tissue salicinoid concentrations \(\geq0.0006\%\) dry weight.

Conclusion: By consolidating and refining existing methods, we developed a reliable, versatile, and more environmentally-friendly procedure for purification and quantification of salicinoids.

Keywords: Flash chromatography, HPLC, phenolic glycoside, poplar, \textit{Populus}, salicinoid.

1. INTRODUCTION

Phenolic glycosides are phytochemicals consisting of a conjugated sugar and phenol aglycone. Salicinoids are a class of phenolic glycosides that are the signature secondary metabolites of the Salicaceae (poplars and willows) [1]. These compounds consist of glucosylated variants of salicyl alcohol, with the simplest one known as salcin (salicyl alcohol linked to \(\beta\)-D-glucopyranose). Isolation and quantification are important for the study of their roles in plant defense against herbivores [1, 2].

Numerous methods exist for extracting, purifying and quantifying phenolic glycosides from plants. These compounds are typically extracted from bark, leaf or fruit tissue into polar solvents, with purification by gel affinity or liquid column chromatography [3-11]. Quality and quantity are typically assessed by high performance liquid chromatography (HPLC) with ultraviolet absorbance or mass spectroscopy detection (based on dominant product ion(s)) [3, 12, 13]; and quality and identity may also be confirmed by nuclear magnetic resonance spectroscopy (NMR) [3, 8-10]. Challenges to measuring phenolic glycosides in plants include separation from complex tissue matrices without contamination or degradation, laborious procedures for purifying standards for instrument calibration, and assurance of reproducible quantification [1, 7, 11].

We consolidated and improved existing methods to create a versatile, combined procedure for purifying analytical standards and quantifying salicinoids in plant foliage. Analytical standards for three abundant salicinoids (Fig. 1) were purified following a three-step approach (Fig. 2): (1) solid-liquid and liquid-liquid extraction, followed by (2) flash chromatography and (3) preparative-scale HPLC. Purity of these standards, and concentrations of salicinoids in \textit{Populus} foliage, were determined using ultra high performance liquid chromatography (UHPLC) with photodiode array (DAD) and single quadrupole mass spectrometry (MS) detection. Our combined procedure facilitates purification and provides multiple options for rapid, reliable quantification of salicinoids in large sets of plant samples.
2. MATERIALS AND METHODS

2.1. Reagents and Materials

The reagent grade solvents used for solid-liquid and liquid-liquid extraction, and the HPLC-grade solvents used for chromatographic separations, were purchased from ThermoFisher Scientific (Waltham, MA, USA). Salicortin (70% purity), HCH-salicortin (83% purity), and tremulacin (96% purity) analytical standards used for UHPLC method validation were previously prepared in our laboratory by solid-liquid and liquid-liquid extraction [5, 6], followed by purification with normal phase liquid chromatography [14].

Foliar source material for bulk extraction and measurement of salicinoids was collected from single ramets of Populus deltoides W. Bartram ex Humphry Marshall (near Arlington, WI, USA), P. tremuloides Michx (near Portage, WI, USA), and P. fremontii S. Watson (near Ogden, UT, USA), and lyophilized or vacuum-dried. Dry foliage was ground with a Wiley Mill (Thomas Scientific; Swedesboro, NJ, USA; 2 mm mesh screen) and stored at -20°C until extraction of salicinoids.

2.2. Extraction of Plant Material for Salicinoid Purification

Salicinoids were extracted and fractionated from three replicate samples from each Populus species using a scaled version of the solid-liquid and liquid-liquid extraction procedure described by Lindroth et al. [6]. Approximately 50 g of dry, ground leaf material was extracted into 300 mL of 4:1 methanol:water (4°C) with sonication for 60 min. Solids were separated by centrifugation for 10 min at 580 g, followed by filtration (Whatman #2 paper) in a Büchner funnel. The filtrate was subjected to sequential liquid-liquid extractions in a 200-mL separatory funnel. First, the filtrate was extracted with 3:1 hexane:chloroform (100, 100, 80, 80, and 80 mL, respectively), retaining the methanol:water layers (bottom). Second, the methanol:water component was extracted with chloroform (200, 120, and 40 mL, respectively), retaining and pooling the chloroform:methanol layers (bottom). The combined chloroform:methanol fraction, containing the salicinoids, was concentrated by rotary evaporation and dried under vacuum.

2.3. Cleanup by Flash Chromatography

The full mass (± 5.5 g) of the mixture containing crude salicinoids was dissolved into 20 mL of 4:1 dichloromethane:methanol, and separated by isocratic flash chromatography (VersaFlash® 40 × 150 mm or 80 × 150 mm silica cartridge, 45-75 μm spherical particles, Supelco, Bellefonte, PA, USA) using 4:1 dichloromethane:methanol at a flow rate of 19 mL min⁻¹ (Sci Log Accu pumping system, Middleton, WI, USA). Fractions (20 mL) were monitored for salicinoids by applying a single drop of each fraction to a silica TLC plate (EM Science, 10 × 10 cm Kieselgel 60 5631/5), separation with 4:1 dichloromethane:methanol, and visual comparison of migration distances with pure standards using an iodine chamber. Fractions enriched in the following compounds were collected as applicable: salicortin (fraction 1), HCH-salicortin (fraction 1), and tremulacin (fraction 2). Fractions were concentrated by rotary evaporation and dried under vacuum. The column was conditioned with ~250 mL of 4:1 dichloromethane:methanol prior to sample separation, and rinsed with ~350 mL of this solvent mixture following separation.

2.4. Purification by High Performance Liquid Chromatography

Dried fractions enriched in salicortin, HCH-salicortin, and tremulacin from flash chromatography were further purified by reverse-phase preparative scale HPLC (Rainin HPXL, France), and assayed for purity by UHPLC-DAD-MS (see below). Each fraction was dissolved into 60:40 water:methanol to a concentration of approximately 500 mg mL⁻¹ and manually injected into the HPLC as sequential 200 μL aliquots. Each injection was separated on an Altex Ultrasphere C₁₈ column (5 μm, 10 mm × 250 mm) equipped with an Altex Ultrasphere guard column (Grace Corp., Columbia, MD) at a flow rate of 3.0 mL min⁻¹ using a gradient of water (with 0.005% formic acid, A) and methanol (B). The following gradient over the course of 45 min was used for purification of salicortin and tremulacin: 0-5 min (80-75% A), 5-25 min (75-50% A), 25-39 min (50-10% A), 39-42 min (10% A), and 42-45 min (10-80% A). A different gradient over the course of 90 min was required to purify HCH-salicortin: 0-5 min (80-77% A), 5-81 min (77-68% A),
81-83 min (68-10% A), 83-87 min (10% A), and 87-90 min (10-80% A). Elution was monitored at 230 nm on a Rainin Dynamax tunable UV detector (Rainin, France), and fractions containing purified compounds were manually collected in 20 mL glass vials. Fractions containing individual compounds were concentrated using a centrifugal vacuum evaporator (Savant SpeedVac Plus SC110A, Waltham, MA, USA), then pooled, vacuum-dried, and assayed for purity by UHPLC-DAD-MS (see below).

2.5. Measurement of Salicinoids by UHPLC-DAD-MS

We evaluated the chemical composition of bulk foliage, as well as the salicinoid content of purified fractions, using UHPLC (Waters Acquity UPLC system, Milford, MA, USA) with diode array detection (DAD; Waters I-class PDA detector) and negative electrospray ionization single quadrupole mass spectrometry (MS; Waters 3100 SQ mass detector) following a modified version of the method from Abreu et al. [15]. Salicinoids in bulk foliage (20-30 mg) were extracted into 1.5 mL of cold (4°C) methanol with sonication (15 min), and salicinoids in purified fractions (0.10-0.50 mg) were dissolved directly into cold methanol. We then diluted, filtered and injected (2 μL) all methanolic salicinoid solutions onto the UHPLC and separated peaks with a Waters Acquity CSH C-18 column (2.1 mm, 1.7 μm) at 40°C with a flow rate of 0.5 mL min⁻¹, using a gradient of water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid. The DAD was configured to scan from 210-400 nm, with 1.2-nm resolution and a sampling rate of 20 points s⁻¹. The operating parameters for negative ionization mass spectrometry were as follows: cone potential, 30 V; capillary potential, 2500 V; extractor potential, 3 V; RF lens potential, 0.1 V; source temperature, 120 °C; desolvation temperature, 250 °C; desolvation gas flow, 500 L h⁻¹; cone gas flow, 10 L h⁻¹; infusion rate, 5 μL min⁻¹; dwell time, 0.025 s.

Quantitative analysis of salicortin, HCH-salicortin, and tremulacin in bulk leaf material was performed using DAD and MS. We introduced salicylic acid-d₃ (100 mg L⁻¹; Sigma-Aldrich, St. Louis, MO, USA) to all samples as an internal standard prior to filtration and injection. For DAD, linear calibration models were fitted to the 274 nm (salicinoids) and 300 nm (internal standard) absorbance wavelengths collected in scanning mode (Table 1). For MS, quadratic calibration models were fitted to the ratios of the negatively charged salicinoid-formate adducts and internal standard parent ions (m/z 141) measured in selective ion recording (SIR) mode (Table 1). Correction factors were applied to address deviations of analytical standards from absolute purity. Instrumental detection and quantification limits were determined as the minimum concentration in a 2-μL injection capable of producing peak-to-peak signal-to-noise ratios of 3:1 and 10:1, respectively.

Identities and purities of salicinoid analytical standards prepared herein were established from the total ion MS chromatograms (TICs, 200-700 m/z). The TIC peak for each new standard was verified to contain the expected two dominant ions (negatively-charged salicinoid and salicinoid-formate adduct ions) at the retention time demonstrated by analysis of existing standards. Purities were reported as the percentage of the total chromatographic peak areas (salicinoid peak area / sum of all peak areas) in each TIC.

2.6. Quality Assurance

We evaluated the effects of foliage extraction efficiency and salicinoid stability in solution on salicinoid recovery. Extraction efficiency was determined by extracting seven P. tremuloides leaf samples into methanol thrice sequentially, and measuring the salicinoid concentration by UHPLC-MS after each extraction; the percent recovery in the first extraction was estimated from the ratio of the amount of salicinoid measured in the first extraction to the summed amounts of that salicinoid recovered in all three sequential extractions. We evaluated salicinoid stability at 22°C in different solvent combinations (pure methanol, pure water, and methanol-

### Table 1. Detection, calibration and reproducibility of salicinoid measurements by UHPLC.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>IDL² (mg L⁻¹)</th>
<th>IQL³ (mg L⁻¹)</th>
<th>MDL⁴ (% dw)</th>
<th>MQL⁵ (% dw)</th>
<th>Calibration Equation⁶</th>
<th>r²</th>
<th>Model SE</th>
<th>Reproducibility (% dw ± SE)⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MS detection</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Salicortin</td>
<td>0.1</td>
<td>0.3</td>
<td>0.0006</td>
<td>0.002</td>
<td>y = 0.0019±0.0092 + 2.55±0.08x – 1.1±0.1x²</td>
<td>0.999</td>
<td>0.017</td>
<td>6.6±0.2</td>
</tr>
<tr>
<td>Tremulacin</td>
<td>0.1</td>
<td>0.4</td>
<td>0.0006</td>
<td>0.002</td>
<td>y = 0.02±0.019 + 3.8±0.2x – 1.4±0.2x²</td>
<td>0.999</td>
<td>0.034</td>
<td>2.11±0.02</td>
</tr>
<tr>
<td>HCH salicortin</td>
<td>0.2</td>
<td>0.4</td>
<td>0.001</td>
<td>0.002</td>
<td>y = 0.035±0.055 + 3.4±0.5x – 1.6±0.6x²</td>
<td>0.981</td>
<td>0.102</td>
<td>n.d</td>
</tr>
<tr>
<td><strong>UV detection</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Salicortin</td>
<td>10</td>
<td>15</td>
<td>0.06</td>
<td>0.09</td>
<td>y = -0.002±0.004 + 0.7±0.01x</td>
<td>0.999</td>
<td>0.009</td>
<td>5.8±0.1</td>
</tr>
<tr>
<td>Tremulacin</td>
<td>10</td>
<td>15</td>
<td>0.06</td>
<td>0.09</td>
<td>y = 0.02±0.02 + 1.4±0.04x</td>
<td>0.993</td>
<td>0.039</td>
<td>2.46±0.05</td>
</tr>
<tr>
<td>HCH salicortin</td>
<td>10</td>
<td>15</td>
<td>0.06</td>
<td>0.09</td>
<td>y = -0.002±0.004 + 0.44±0.01x</td>
<td>0.994</td>
<td>0.010</td>
<td>n.d</td>
</tr>
</tbody>
</table>

² Minimum instrumental detection limit (peak-to-peak S/N = 3).
³ Minimum instrumental quantification limit (peak-to-peak S/N =10).
⁴ Minimum analyte detectable by this method as a percentage of dry plant tissue weight (% dw), based on (a).
⁵ Minimum analyte quantifiable by this method as a percentage of dry plant tissue weight, based on (b).
⁶ Instrument calibration equation ±SE for 15 – 750 mg L⁻¹ (15, 30, 150, 375, 750 mg L⁻¹, duplicated at each level) and method reporting range (0.1 - 10 % dw).
⁷ N = 8, averaged across 1.5 days.
extracted leaf tissue matrix) prepared in polypropylene
HPLC vials capped with rubber/PTFE septa. Analyte con-
centrations were measured initially and at 4-6 time points
(3, 5, 10, 25, 50, 75 h) using UHPLC-MS; concentration
changes across all time points were evaluated using repeated
measures analyses of variance (JMP11.0, SAS Institute,
Cary, NC, USA).

We evaluated the effect of quantifying salicinoids based
on one or both of the dominant ions formed during negative
electrospray ionization MS (deprotonated and formate ad-
duct). Specifically, we compared salicinoid concentrations
measured in 28 injections repeated over time that were calcu-
lated from a single ion (formate adduct) and summed ions
deprontated and formate adduct) to identify the effects of
each approach on sensitivity and reliability.

Percent recovery of each purified salicinoid was calculated
as \( R = 100 \times \left( \frac{M_1 \times C_2}{M_1 \times C_1} \right) \), where
\( M_1 = \) dry mass (g) of source foliage before purification
\( M_2 = \) dry mass (g) of purified salicinoid
\( C_1 = \) concentration (g·g\(^{-1}\)) of salicinoid in source foliage be-
fore purification
\( C_2 = \) concentration (g·g\(^{-1}\)) of salicinoid in purified material

3. RESULTS AND DISCUSSION

3.1. Bulk Extraction for Salicinoid Isolation

Obtaining a crude salicinoid extract from 50 g of dry fo-
lage using solid-liquid and liquid-liquid extraction (Fig. 2)
required ~3 hours, 240 mL of methanol, 330 mL of hexane,
and 470 mL of chloroform. Recoveries of salicinoids ran-
ged from 8 to 100%, depending on compound and source sp-
cies.

3.2. Flash Chromatography for Cleanup of Crude Salici-
noid Extracts

Use of a sealed flash chromatography system (Fig. 2)
with manufactured columns improved upon conventional,
packed column techniques employed by Keeover-Ring et al.
[3] and Still et al. [14], by increasing separation speed and
decreasing operator exposure to silica dust and toxic sol-
vents. Passing crude extracts (~5.5 g in 20 mL) through an
80 × 150 mm column yielded fractions enriched in salici-
noids ≤75%, and required ≤2 L of solvent mix (1600 mL
dichloromethane, 400 mL methanol) and <2 hours of opera-
tor time per aliquot.

3.3. Preparative HPLC for Purification of Salicinoids

Further separation of these enriched fractions by HPLC
(Fig. 2) achieved salicinoid purities ≤99% (Table 2; Fig. 3).
This HPLC method improved upon earlier work by Keeo-
ver-Ring et al. [3] by replacing acetonitrile with methanol in
the mobile phase (increasing safety and decreasing costs and
environmental impact) and optimizing gradient elution to
resolve multiple salicinoids. Salicortin and tremulacin purifi-
cation required 45 min and yielded ~10-50 mg per injection;
HCH-salicortin purification required 90 minutes per injec-
tion and yielded ~5-10 mg per injection.

![Flowchart showing the extraction and purification process.]

Table 2. Recovery efficiencies\(^a\) and purities\(^b\) for dominant salicinoids in three Populus species.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>P. tremuloides</th>
<th></th>
<th>P. fremontii</th>
<th></th>
<th>P. deltoides</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery</td>
<td>Purity</td>
<td>Recovery</td>
<td>Purity</td>
<td>Recovery</td>
<td>Purity</td>
</tr>
<tr>
<td>Salicortin (fraction 1)</td>
<td>6-23%</td>
<td>90-99%</td>
<td>10-30%</td>
<td>90-&gt;99%</td>
<td>14-28%</td>
<td>72-91%</td>
</tr>
<tr>
<td>HCH-salicortin (fraction 1)</td>
<td>n/a(^c)</td>
<td>n/a</td>
<td>11-28%</td>
<td>79-99%</td>
<td>15-63%</td>
<td>58-87%</td>
</tr>
<tr>
<td>HCH-salicortin (fraction 2)</td>
<td>n/a</td>
<td>n/a</td>
<td>4-9%</td>
<td>79-92%</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Tremulacin (fraction 1)</td>
<td>19-21%</td>
<td>97-98%</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Tremulacin (fraction 2)</td>
<td>13-27%</td>
<td>87-98%</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

\(^a\) Percent mass of available analyte in leaf source material recovered in purest form. Species × fraction combinations yielding highest analyte purities shown in bold.

\(^b\) Percentage of analyte in fraction, by MS-TIC peak area after preparative HPLC.

\(^c\) Cleanup of crude extracts by flash chromatography yielded two solvent fractions. Fraction 2 did not contain appreciable amounts of salicortin.

\(^d\) not applicable.
Purification and Analysis of Salicinoids

Purification and analysis of salicinoids using flash chromatography increased HPLC throughput by decreasing injection size and eliminating viscous/insoluble materials. Large HPLC column loadings were characterized by diffusive peak broadening, but feasible when the injected material was sufficiently soluble, contained relatively high concentrations of salicinoids, low concentrations of impurities, and when the retention times of the impurities were very different from the retention times of the salicinoids. The rate of purifying low abundance salicinoids from complex mixtures was limited by the amount of salicinoid in each injection, because smaller injection volumes and longer run times were needed to achieve satisfactory separation.

Purification of HCH-salicortin by HPLC was complicated by coelution of up to four other compounds (m/z 423, m/z 431, m/z 453, and m/z 463). Adjusting the mobile phase composition doubled the HPLC runtime (from 45 to 90 min) and achieved partial separation, allowing higher purity HCH-salicortin to be collected from the tail of its broad peak at ~75 min (at the expense of recovery). An alternative extraction procedure may facilitate purification of HCH-salicortin; Rehill et al. [5] isolated HCH-salicortin from P. fremontii foliage using acetone extraction and purification with a single flash chromatography step, and subsequent UHPLC-DAD-MS analysis of this product revealed <1% contamination.

3.4. Quantification of Salicinoids in Plant Tissue by UHPLC

Compared with analytical HPLC, UHPLC exploits smaller diameter column packing resins and higher operating pressures to decrease separation times and increase chromatographic resolution. We modified the UHPLC method of Abreu et al. [15] by adjusting the mobile phase gradient to improve analyte separation, and demonstrated reproducible quadratic mass calibrations for quantification by DAD and MS (Table 1). Negative electrospray ionization MS was approximately 100× more sensitive than DAD. Measurements of salicortin and tremulacin in P. tremuloides extracts by DAD were slightly lower and higher, respectively, than by MS, indicating that a single detector type should be used to insure optimum comparability of data.

We used quantitative UHPLC-MS to evaluate the salicinoid content of Populus leaf samples prior to bulk salicinoid isolation. Populus tremuloides contained abundant salicortin (9.6% dw) and tremulacin (4.3% dw), and P. fremontii and P. deltoides both contained relatively large amounts of...
salicortin (3.5 and 6.8% dw, respectively), and HCH-salicortin (1.9 and 3.2% dw, respectively). Incorporation of internal standardization (Abreu et al. [15]) into quantitative analysis enabled reproducible measurements by DAD and MS over the course of days, while filtration (Rubert-Nason et al. [13]) decreased risk of UHPLC clogging by particles in plant extracts (Table 1).

3.5. Quality Assurance

Multiple variables can potentially affect salicinoid purification and measurement, including extraction efficiency, hydrolysis [7, 16, 17], and sorption to surfaces. Single methanol extractions consistently recovered 92 ± 1% (SE) of each salicinoid from ground foliage, indicating suitability of single extractions for obtaining salicinoids (provided that quantitative measurements are corrected for recovery efficiency). Analyte concentrations did not change significantly over 3 days at room temperature (22°C) in various combinations of water, methanol, and extracted P. fremontii leaf matrix (pH range 4.6 – 7.4), indicating that degradation and sorption did not meaningfully impact recoveries over the much shorter time periods used in our standard method (provided that hydrolytic plant enzymes [17] were initially denatured, as herein). However, analyte losses sometimes occur in methanolic plant extracts over longer time periods [13], and may be accelerated by high temperatures [7, 16, 17], leading us to recommend that extracts for quantitative analysis be stored at < 10°C and analyzed within ≤ 2 days [13].

Single quadrupole MS response magnitudes can vary in response to environmental fluctuations, and environmental effects may vary depending on MS operating conditions. To determine the effect on salicinoid quantification from using one or both dominant ions formed during single quadrupole MS (i.e., the negatively charged salicinoid and salicinoid-formate adduct ions), we compared both approaches in terms of instrument sensitivity and measurement reproducibility. Summing both ions increased sensitivity by 27% and decreased propensity for calibration drift without impacting the quality of calibration curves (data not shown), indicating that quantification should use the sum of both ions rather than a single ion.

CONCLUSION

We present combined procedures for purifying salicinoids from Populus foliage and quantifying them with multiple detection modes, and provide guidelines for quality assurance. Consolidation and refinement of existing methods facilitates isolation of novel salicinoids, and insures reliable quantification for investigations of the biological effects of salicinoids. The improved purification procedure mitigates occupational and environmental hazards through better-containment of halogenated solvents during flash chromatography and substitution of less toxic solvents in preparative HPLC. Diode array and single quadrupole MS detection (used in conjunction with UHPLC, internal and process control standards) are both viable options for quantitative analysis, with the former being more affordable and widely available and the latter being more sensitive and selective.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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ACKNOWLEDGEMENTS

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REFERENCES


