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Anti-inflammatory Activity of Salicylanilide Compounds from Gondopuro Oil as an In-Vitro

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Abstract: *A component of gondopuro oil is methyl salicylate, which contains functional groups amenable to chemical modification. Functional group transformations can occur through various reactions, including aminolysis, to yield compounds such as salicylamide or salicylanilide. The precursor for synthesizing these amide derivatives is a carboxylic acid, utilizing natural methyl salicylate from gondopuro oil and amines. This study employed sonochemical methods for 3, 4, and 5 hours with temperature control at approximately 60ºC. Liquid extraction was performed using hexane and distilled water and repeated 2 to 3 times until two phases formed. The mixture was then left overnight at a temperature below 10ºC and decanted. Subsequent extraction was conducted with 10 mL of cold 5% NaOH twice, forming two phases: the n-hexane fraction and the NaOH fraction. The NaOH fraction was heated using a water bath to evaporate the solvent, yielding a solid that further dried in an oven at 40ºC to form crystals. The synthesized crystals were dried in an oven at 40ºC, and the yield of the synthesized powder was calculated. The results indicate that salicylanilide compounds can be synthesized using sonochemical methods, with the highest yield of 11.23% obtained from the third sample after 5 hours. Furthermore, salicylanilide compounds exhibited anti-inflammatory activity at a concentration of 100.0 ppm, achieving 46.07% inhibition.*

Keywords*: Anti-inflammatory, Gondopuro oil, Inhibition, Salicylanilide, Sonochemistry*

INTRODUCTION

Gondopura oil is a class of essential oils widely known by the general public. The main component of gondopuro oil is 96-99% methyl salicylate (Ma'mun, 2015)(Sulistyo et al., 2015). This compound is widely used in the pharmaceutical, fragrances, and food and beverage industries. Apart from that, the methyl salicylate compound has many properties so that it can be used as an anti-inflammatory, acute rheumatic medicine to relieve pain and breathing, anti-inflammatory and analgesic (F. et al., 2014) (Ripa et al., 2015) (Of et al. 2015).

Methyl salicylate (o-hydroxymethyl benzoate) has functional groups that allow it to be converted into other compounds derived from methyl salicylate through changes in functional groups through organic chemical reactions. Changes in functional groups can occur through chemical reactions, including through the hydrolysis reaction to produce salicylic acid, through the aminolysis reaction to produce salicylamide or salicylanilide, through the transesterification reaction to produce other ester compounds such as ethyl salicylate, isoamyl salicylate, phenyl salicylate (salol), etc (Hendrata, 2014) (Retnowati & Azzuhro, 2013). Other reactions that can change the functional groups in methyl salicylate include alkylation, reduction, and electrophilic substitution on the benzene ring (Retnowati & Azzuhro, 2013).

The sonochemical method is an alternative to conventional stirring with low energy and efficient results (Draye et al., 2020) (Indriyanti & Prahasiwi, 2020). This method is also effective for synthesizing compounds in a relatively short time. This research also uses a Decyclocarbodiimide (DCC) coupling reagent to speed up the coupling process of a reaction (Farshori et al., 2010).

Based on the above background, the researcher intends to synthesize amide derivatives using a sonochemical method for 5 hours. Tests for the purity of synthetic compounds include melting point tests, solubility tests, and thin-layer chromatography tests. Identification of synthetic compounds by spectrum elucidation using infrared spectrophotometry (FTIR-ATR), GCMS, and in vitro anti-inflammatory activity testing.

RESEARCH METHODS

Materials and Tools

The ingredients used in this research were gondopuro oil, aniline (p.a), ethanol (p.a), distilled water, methanol (p.a), and Bovine serum albumin (BSA). The tools used in this research are glassware in the laboratory, sonicator, IR spectrometer, spectrophotometer, melting point apparatus, GC-MS, vacuum rotary evaporator, Buchner filter, TLC plate, UV spectrophotometer -Vis, melting point apparatus and capillary tube.

Procedures

Synthesis of Salicylanilides

12 mL of gondopuro oil was reacted with 9 mL of aniline, 50 mL of hexane, and 10 mL of NaOMe10% sonicated for 3, 4, and 5 hours at 600C. Then, liquid extraction was carried out using 50 mL of hexane and distilled water 2 to 3 times until 2 phases were formed. These were then separated in a separating funnel into a hexane fraction and a water fraction. The n-hexane fraction was added with anhydrous Na2SO4, then left for one night at a temperature of less than 100C and decanted. They were then extracted with 10 mL of cold 5% NaOH 2 times until two phases were formed, namely the n-hexane and NaOH fractions. The NaOH fraction obtained is then heated using a water bath to evaporate the solvent so that it becomes a solid, which is then placed in an oven to form crystals. The crystals obtained were then dried using an oven at 40ºC until dry, and then the yield of the synthesized powder was calculated (Sulistyo et al., 2015) (Sondhi et al., 2009).

Structure elucidation with Infrared Spectroscopy (FTIR-ATR) and GC-MS

The synthesized compound was placed on an ATR crystal and its spectrum was measured at a wave number of 4000-500 cm^{-1} and structure elucidation with GC-MS

Anti-Inflammatory Test by In-vitro Activities

In vitro anti-inflammatory test based on the research method by (Williams et al., 2008). Stages testing activity of synthesis results in the denaturation of Bovine Serum Albumin (BSA). Five ml positive control solution comprised 4.950 l BSA and 50 l diclofenac sodium solution. The control solution was made in various concentrations, namely 100 ppm, 10 ppm, one ppm, and 0.1 ppm. Each solution was vortexed and then incubated for 30 minutes at room temperature (27ºC). After that, it was heated for 5 minutes at 72ºC. Then, it was left at room temperature (27ºC) for 25 minutes. Then, the turbidity was measured using a UV–Vis spectrophotometer at a wavelength of 660 nm (Almira et al., 2021). The percentage of inhibition of BSA denaturation can be counted with this formula.

$$\%inhibition = \frac{Abs\ Negative\ Control - Abs\ Sample}{Abs\ Negative\ Control} X\ 100\%$$

RESULTS AND DISCUSSION

The synthesis of salicylanilide compounds in this study was carried out based on the research method (Retnowati & Azzuhro, 2013) with slight modifications in sonochemical methods. The resulting compound was tested for FTIR-ATR and GCMS and potential as an anti-inflammatory activity in vitro. The physical properties of Gondopuro oil were determined and compared with the standard material safety data sheet (MSDS) for wintergreen oil shown in Table 1.

Table 1. Physical Properties of Gondopuro Oil

Physical Properties Parameters	Gondopuro Oil	Standard wintergreen oil (MSDS)
Being	Liquid	Liquid
Colour	Pale yellow	Colourless to yellowish
Smell	Sharp	-
Specific gravity	1.185 g/mL	1.18 g/mL

Table 1 shows that the form, color, and specific gravity of the gondopuro oil used in this research are close to the standard data contained in the material safety data sheet (MSDS) for wintergreen oil. Gondopuro oil was identified using FTIR to determine functional groups and GC-MS to determine synthesized compounds' chemical structure and abundance. This analysis can also be used to compare the FTIR and GC-MS analysis results of reaction compounds.

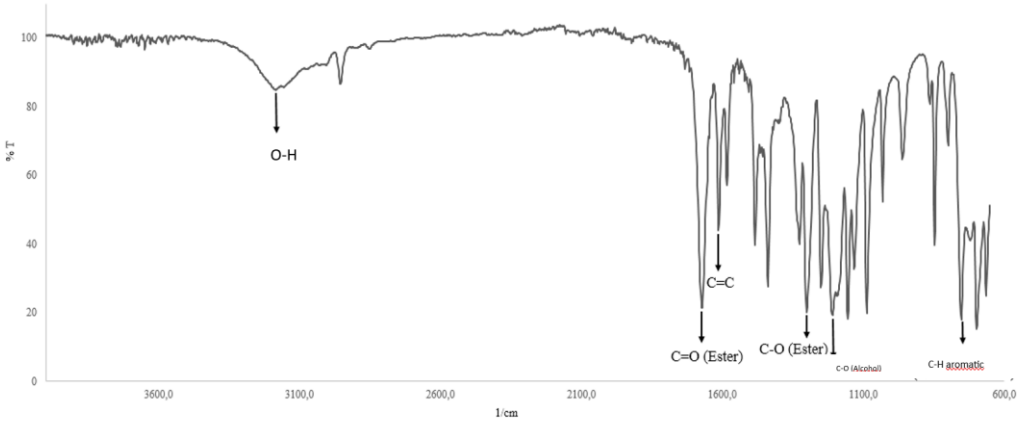

Figure 1. FTIR Spectra Graph of Gondopuro Oil

The results of the spectrum analysis of gondopuro oil using FTIR in Figure 1 show that there is vibration absorption in the 3185 cm-1 area, namely stretching vibration =C-H sp2 (aromatic ring), which is supported by absorption in the 753 cm-1 area, namely bending vibration =C-H. The absorption in the 1674 cm-1 area is the C=O stretching vibration for esters. The functional group is supported by solid absorption in 1301 cm-1 and 1251 cm-1

areas, namely, C-O (ester) stretching vibrations, shown by two peaks between 1300-1000 cm-1. Absorption appears in the 1584 cm-1 area, namely vibration stretch C=C for the aromatic ring. The absorption in the 1210 cm-1 area is the C-O stretching vibration for alcohol.

Identification using GC showed a peak at a retention time of 6,103 minutes with an abundance of 99.97%, showing a mass spectrum with a molecular weight of 152, which indicated that the compound was a methyl salicylate compound. The GC-MS test results for gondopuro oil are shown in Figures 2 and 3.

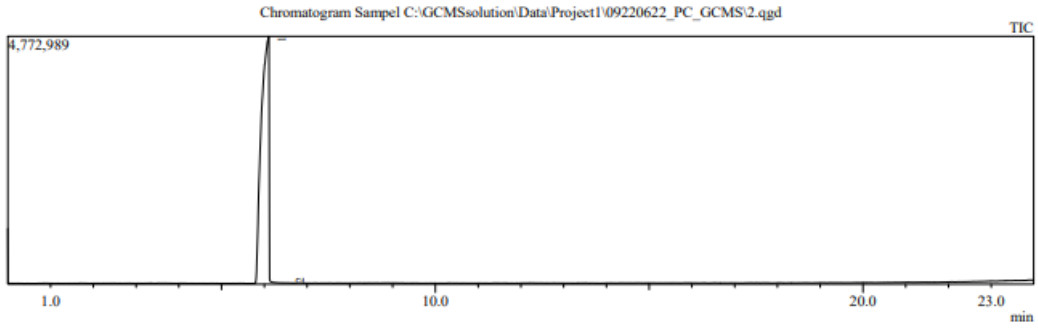

Figure 2. Chromatogram of Gondopuro Oil

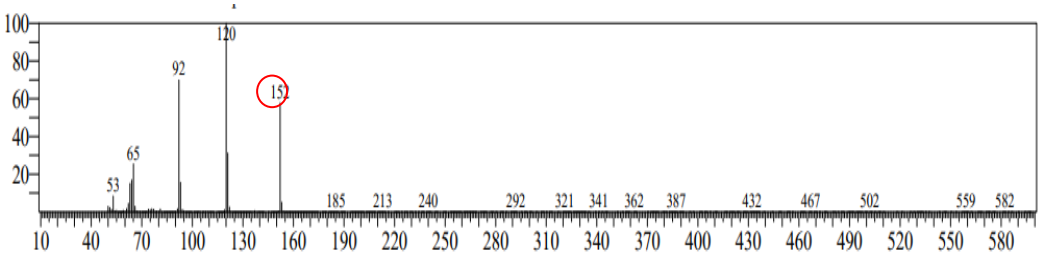

Figure 3. Mass spectrum of Gondopuro oil with a retention time of 6,100 minutes

Mass spectra results from GC-MS testing of gondopuro oil showed that the fragmentation pattern of one of the components of gondopuro oil included m/z = 121 due to the loss of the CH3O radical ion (M-31), followed by the release of the CO carbonyl radical ion (M-31-28) producing a peak. m/z = 93. Peak m/z = 93 produces peak m/z = 64 (M-29) due to the loss of CHO radical ions. The peak m/z = 51 (M-13) occurs from m/z = 65, which loses CH radical ions. The fragmentation pattern of the methyl salicylate compound can be seen in Figure 4.

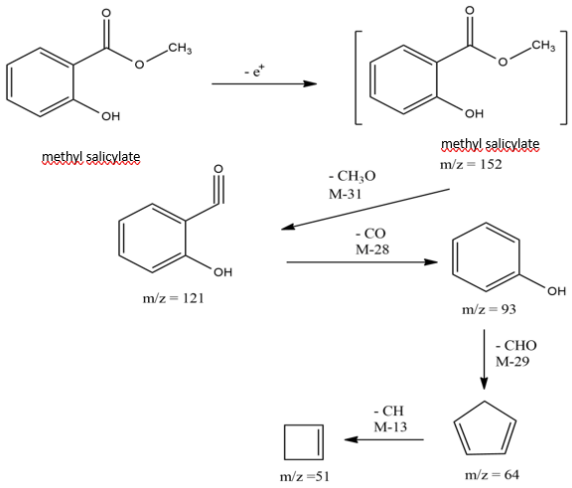

Figure 4. Fragmentation Pattern of Methyl Salicylate Compound

Salicylanilide can be synthesized directly by reacting methyl salicylate from gondopuro oil with aniline solvent. Aniline is a nucleophile, so the N atom in aniline can attack the C carbonyl atom, which is double bonded to the O atom in methyl salicylate. This process causes a transitional attachment between aniline and methyl salicylate, where the N: atom of aniline will experience attachment to the C atom in methyl salicylate, and the –OCH3 group will experience a bond cleavage from methyl salicylate. The process is then continued with the release of the H atom, which is bound to the N atom in aniline as the primary amine and forms an amide group, thus obtaining the salicylanilide compound. The synthesis of salicylanilide was carried out via a nucleophilic substitution reaction using the sonication method. If highly reactive intermediate compounds are excessively activated, intramolecular reactions will occur in the intermediate compound itself. It is intended to shorten the synthesis time, where ultrasonic wave radiation can speed up the reaction. Ultrasonic waves in a liquid medium can cause acoustic cavitation (Manickam et al., 2023). During the cavitation process, bubble collapse will occur (bubble instability), namely the breaking of tiny bubbles due to sound. The synthesis results can be seen in Table 4.

Table 4. % Yield from Synthesis Results

Sample	Theoretical Weight (g)	Weight of Synthesis Results (g)	% *yield*
Sample 1 (3 Hour)	1.4841	0.1058	7.13
Sample 2 (4 Hour)	1.4841	0.1316	8.87
Sample 3 (5 Hour)	1.4841	0.1712	11.53

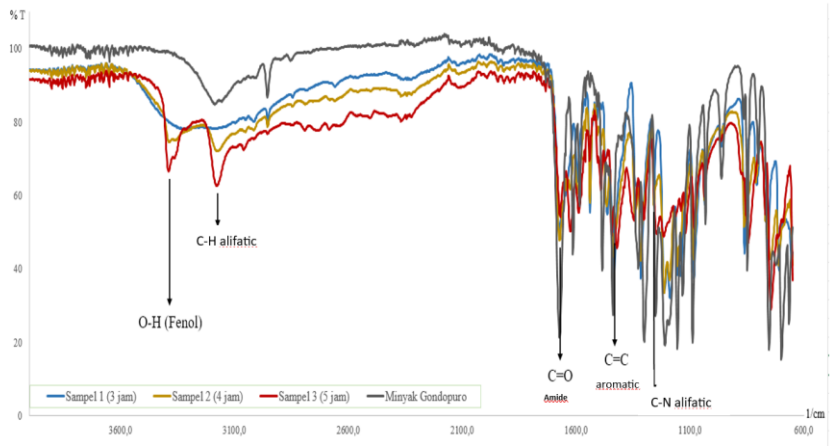

Figure 5. FTIR Spectra Graph of Reaction Result Compounds

Table 5. Data on Wave Numbers of Reaction Result Compounds

Number	Group	Reaction Result Compounds			References (Suratmo dkk., 2013)
		Sample 1	Sample 2	Sample 3	
1	O-H (phenol)	3347	3386	3390	3406.05
2	C-H aliphatic	3161	3051	3176	2991.39
3	C=O (Amide)	1674	1627	1588	1631.67
4	C=C aromatic	1433	1435	1444	1458
5	C-N aliphatic	1316	1258	1316	1253.64

Based on the wave number data in Table 5 and the FT-IR spectrum in Figure 5, it shows that there is absorption in the 3390 cm-1 area, namely the O-H stretching vibration of phenol, which is supported by absorption in the 1444 cm-1 area, namely the aromatic C=C stretching vibration. Next, absorption appears in the 3051 cm-1 area, which is the aliphatic C-H stretching vibration. The absorption for C=O (amide) is shown in the 1674 cm-1 area, while the absorption in the 1258 cm-1 area is the aliphatic C-N stretching vibration. Amides have a typical absorption in the 1680-1630 cm-1 region, namely C=O stretching vibrations. The results of the FTIR test of the reaction compound show that the wave number of the synthesized compound is close to the wave number in the research (Retnowati & Azzuhro, 2013).

The resulting compound was tested for anti-inflammatory activity using Bovine Serum Albumin (BSA). Protein denaturation is when proteins lose their tertiary and secondary structures due to external compounds such as strong acids, bases, organic salts, organic solvents, and heating. Generally, proteins lose their biological function when they are denatured. According to (Whittaker Vogler, 2008), compounds with a percentage of protein denaturation inhibition of >20% have anti-inflammatory activity. Testing of anti-inflammatory activity on protein denaturation was carried out by adding BSA solution to reduce the use of live specimens in the drug development process. When BSA was heated, BSA denaturation would occur.

According to Nasution et al. (2019), compounds capable of stabilizing proteins against denaturation have potential anti-inflammatory properties. This is due to interactions between bovine serum albumin (BSA) and the active substances of these compounds, resulting in the binding of the active substances with amino acids such as tyrosine and lysine. However, it is noted that when the active substance adheres to itself, it does not

prevent BSA denaturation. An anti-inflammatory test utilizing protein denaturation was conducted on the synthesized compound, with Diclofenac Sodium serving as the positive control.

Table 6. In Vitro Anti-Inflammatory Test Results

Number	Sample	Concentration (ppm)	Absorbance	% Inhibition
1	Negative Control	-	0.280	-
2	Na diklofenac	0.1	0.210	25.00
		1.0	0.180	35.71
		10.0	0.098	65.00
		100.0	0.082	70.71
3	Salicylanilide	0.1	0.232	17.14
		1.0	0.194	30.71
		10.0	0.177	36.79
		100.0	0.151	46.07

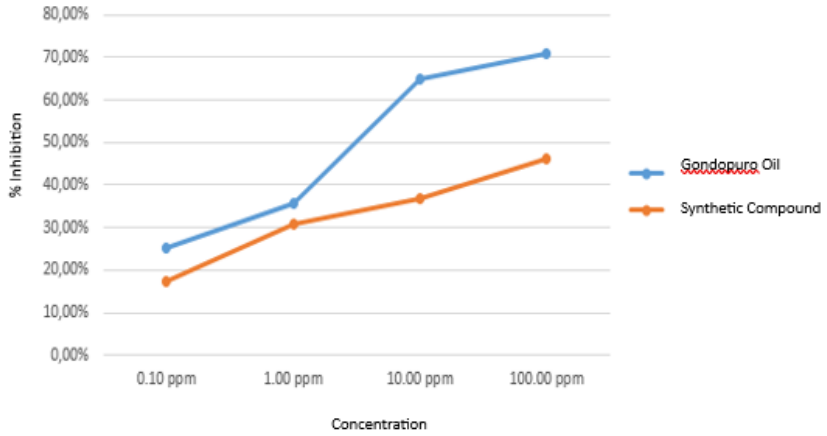

Figure 7. Graph of % Inhibition of Anti-Inflammatory Tests in Vitro

The results of the anti-inflammatory test for Diclofenac Sodium showed that the activity inhibited protein denaturation with a percent inhibition of 25.00% at a concentration of 0.1 ppm and increased as the concentration increased to 1.0 ppm; 10.0 ppm and 100.0 ppm 35.71%, 65.00%, and 70.71%. The results of anti-inflammatory testing of the reaction compound showed activity to inhibit protein denaturation starting from a concentration of 1.0 ppm, namely with a % inhibition of 30.71%. The compound resulting from the reaction at a concentration of 0.1 ppm showed a % inhibition of 17.14%, which means that at this concentration, it did not have an anti-inflammatory effect; this is because the % inhibition increased with increasing concentration so that at a concentration of 10.0 ppm it had a % inhibition of 36.79 % and at a concentration of 100.0 ppm it has an inhibition % of 46.07%. In vitro, anti-inflammatory testing results show that the reaction compound has anti-inflammatory activity because it has a % inhibition value of >20%.

CONCLUSIONS

Salicylanilide compounds can be synthesized using sonochemical methods, with the highest yield observed in the third sample after 5 hours, achieving 11.23%. These compounds exhibit anti-inflammatory activity, demonstrated by a 46.07% inhibition at a concentration of 100.0 ppm.

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