

LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROSCOPY ASSAY FOR QUERCIN AND CONJUGATED QUERCETIN IN SKIN RODS (*Hibiscus tiliaceus*)

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**LIQUID CHROMATOGRAPHY-TANDEM MASS
SPECTROSCOPY ASSAY FOR QUERCIN AND CONJUGATED
QUERCETIN IN SKIN RODS (*Hibiscus tiliaceus*)**

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ABSTRACT

Waru plants have efficacy as medicine for smoothing urination, fertility signs, coughing and fever. This study aims to isolate and identify compound markers from the skin of waru stem as one of the parameters of standardization of natural medicine using the *Quarsetin* comparison. In this study, the extraction of waru bark was carried out using the maceration method using methanol solvent. Marker compounds detected were isolated using preparative TLC and isolates were identified using liquid chromatography-mass spectrometry (LC-MS). Compounds detected as marker compounds on waru bark are found at hRf 0.73. The results of the identification of the structure of the marker compound isolates using Liquid Chromatography-Mass Spectrometry showed that the compound on the bark of waru (*Hibiscus tiliaceus*) is a quartet in derivative flavonoid compound that is the compound Quercetin 3-O- (6 " - acetyl-glucoside) with a relative molecular mass of 507 , 3483 m / z.

Keywords: Waru bark, identification of marker compounds, LC-MS

INTRODUCTION

Marker compounds are used as a comparison of the presence of a plant extract in traditional medicinal products. The indicator used to guarantee the quality of traditional medicines is by analyzing marker and qualitative and quantitative compounds. Searching for marker compounds can also be applied to the process of determining the authenticity of species, finding new sources or replacing raw materials, optimizing extraction methods, purification, structural elucidation and determination of purity.

The waru plant (*Hibiscus tiliaceus*) is one of the tropical plants that is often found growing on the coast or coastal areas such as Gorontalo. Waru (*Hibiscus tiliaceus*, which belongs to the tribe of *Malvaceae*), also known as Waru the sea. Waru (*Hibiscus tiliaceus*) plants also often grow wild in forests, fields and home yards that are used as protective trees. The use of the Waru (*Hibiscus tiliaceus*) plant as a fever-lowering drug is based on empirical experiences that have been handed down from generation to generation by North Gorontalo people in Kec. Bualemo. According to Dalimartha (2004) Waru plants (*Hibiscus tiliaceus*) can be used as cough medicine, tonsillitis, inflammation of the intestine, dysentery and so on. Waru plants have chemical constituents, among others: saponins, flavonoids, and polyphenols which are included in secondary metabolites that are antioxidative. Based on the description above, it is necessary to conduct research to determine the compound marker (marker) of secondary metabolites that have bioactivity properties of methanol extract of Waru stem skin (*Hibiscus tiliaceus*) using the method of Liquid Chromatography-Mass Spectrometry (LC-MS).

METHOD

Place and time of research

The research was conducted at the Phytochemical Laboratory, Department of Pharmacy, Faculty of Sports and Health, Gorontalo State University in February to June 2018.

Tools and materials Tool. Stirring rod, porcelain saucer, watch glass, horn spoon, drip pipette, measuring cup, beaker, funnel, analytic balance, Ohaus balance, hot plate, filter, filter paper, macerator oven, aluminium foil, capillary pipe, separating funnel, stand clamps, pyrex tubes, chamber, and LC-MS material

The materials used in this study were waru bark, methanol, n-hexane, ethyl acetate, sulfuric acid, TLC plate, KLTP plate, mineral water and acetone.

Research methods

The ingredients of the waru bark (*Hibiscus tiliaceus*) are obtained from Boalemo Village, North Gorontalo Regency, Gorontalo Province Plant Determination Waru (*Hibiscus tiliaceus*) plants are identified or determined at the Natural Materials Laboratory, Department of Pharmacy, Faculty of Sports and Health, Gorontalo State University

Waru's Bark Extraction Waru bark that has been chopped, extracted with using the maceration method by inserting 500 g of Simplicial hazel into a macerator

container then adding methanol ± 1000 ml, the extraction process lasts for 3 x 24 hours accompanied by stirrer during extraction time.

Rendamen

The *rendamen* from the methanol extract of the bark of waru (*Hibiscus tiliaceus*) bark is then calculated using the formula

Phytochemical screening

Detection of compounds using spray reagents was carried out to find out what group compounds were found in the patches of chromatogram. Detection is carried out on all types of compounds with reagents.

Alkaloids

As much as 0.1 gr of the thick extract of waru bark, which has been dissolved in methanol, added 5 mL of 10% HCl and divided into 3 test tubes. The presence of alkaloid compounds when there is a white precipitate with Mayer reagents, orange-red deposits with Dragendorf reagents in the sample (Edeoga et al, 2005).

Flavonoids

A total of 0.1 gr of the thick extract of waru bark which has been dissolved in methanol is then divided into four test tubes. The first tube is used as a control tube, the second, third, and fourth tubes are respectively added NaOH, concentrated H₂SO₄, and concentrated Mg-HCl powder. The colour of each tube is compared to a control tube if there is a change in colour it is positive to contain flavonoids (Harborne, 2008 in Taher, 2011).

Saponin

As much as 0.1 gr of the thick extract of waru bark are added 5 mL of hot distilled water, cooled, and shaken vigorously for 10 minutes. Saponins are present when a solid foam is formed.

Tanin

As much as 0.1 gr of the thick extract of the skin of waru stem is extracted which has been dissolved with methanol and 5 ml of mineral water. The filtrate obtained then added a few drops of FeCl₃ 1%. The presence of tannin compounds is shown by the formation of green, blue or purple (Edeoga et al, 2005).

Liquid-Liquid Partition

Liquid-liquid partitioning is an extraction method that uses a separating funnel and a ratio of two solvents which do not mix together in the extraction process. In this study the solvent used in the liquid-liquid partition process was n-hexane. The thick extract of waru skin which has been dissolved with methanol (*Hibiscus tiliaceus*) is put into a funnel, and N-hexane is added with a certain ratio, then it is vortexed and left for several minutes. After forming two layers, the bottom layer (methanol) is taken. The results obtained at this stage were continued in the next process, thin layer chromatography (TLC).

Thin layer chromatography

Methanol thick extract of the skin of waru stem (*Hibiscus tiliaceus*) which has undergone a phase of separation with using the liquid-liquid partition method,

the next step before isolating the compound, an eluent mixture was tested using TLC, to test the eluent according to the right ratio. The elution results were observed under ultraviolet (UV) 254 nm and 366 nm.

Vacuum Liquid Chromatography

Vacuum Liquid

Chromatography (KCV) is a method of separating compounds based on the compounds to be separated which are distributed in the stationary phase and the mobile phase with different comparisons (Sastrohamidjojo, 1985). At this stage, the methanol extract of the bark of waru bark is separated by using several mobile phase comparisons including n-hexane 100%, n-hexane: methanol with a certain ratio and 100% methanol.

Isolation of Compounds Markers with KLTP (Preparative Thin Layer Chromatography)

In this study, the isolation of compounds from the bark of waru (*Hibiscus tiliaceus*) was carried out.

with the preparative TLC method. The tape produced by preparative TLC which is suspected to be a marker compound on the Waru bark (*Hibiscus tiliaceus*) is scraped and dissolved in the appropriate solvent and then tested for its purity by TLC. The purity test of the isolate which is thought to be a marker compound was carried out by TLC using different mobile phases, and silica gel 60 F 254 was used as the stationary phase. After development is complete, spots are observed in visible light, 254 nm UV light and 366 nm UV light. Isolates dissolved in methanol then taken 2-3 mL, inserted in cuvettes and measured spectrum at a wavelength of 200-500 nm. The blank used is methanol. Identification is done using sliding reagents. The reagents used were reagents of sodium methoxide, sodium acetate anhydrous powder, AlCl₃, and H₃BO₃ powder.

Identification with LCMS / MS LCMS (Liquid Chromatography and Mass Spectrometry) The isolation results of the marker compound from the methanol extract of waru bark (*Hibiscus tiliaceus*) using preparative thin layer chromatography (KLTP) which had been scraped were then identified using Liquid Chromatography-mass Spectrometry (LC-MS).

LC-MS uses a mobile or solvent phase to carry samples through a column. Separation of samples is done by using a C18 column. Then the sample will be eluted using a pump. The elution process uses 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile

solution (B). After 25 minutes, 90% of solution A and 10% of solution B will gradually change to 30% solution A, and 70% solution B. The injection volume is 10 µL, then the sample below leads to a mass spectrometer (ESI) for further determination. qualitatively with a flow rate of 0.3 ml/minute, the capillary temperature is set at a temperature of 50 spray C, the ion spray voltage is 4 kV and the capillary voltage is 36 v (Lee, 1999)

u stem Skin Extraction

Table 01 Rendamen Results Obtained

Sample Weight (gram)	Solvent (mL)	Extract Weight (gram)	Rendamen (%)
500	1000	50	10

Table 01. shows that the extraction of waru bark (*Hibiscus tiliaceus*) using maceration method with sample weight 500 gram and using a solvent as much as 1000 mL, producing as much as 50 grams of the extract with 10% per cent rendamen.

Waru Stem Skin Phytochemical Screening

Table 02. Results of phytochemical screening Compound

Reactor		Test results	Information
Flavonoids	NaOH		Orange Positive (+)
Alkaloids	Mayer Wagner Dragenroff	There is no sediment	Negative (-)
Tanin	FeCl ₃	Blackish green	Positive (+)
Saponin		Hot water Foaming	Positive (+)

Table 02. shows the results of phytochemical screening from the skin of waru stems, where the identification of flavonoid compounds with NaOH reagents obtained positive results and yellow changes occurred. Alkaloid compounds give negative results because they don't

the presence of deposits in the extract is reacted. For tannin compounds when reacted with reagents FeCl₃ gives a colour change to blackish green, as well as saponins which when added to water and heated to produce foam id-liquid partition

Table 03. Liquid-Liquid Partition Results

No	Solvent	Extract Weight	Extract color
1.	N-hexane dissolves	10 grams	Brownish yellow
2.	Soluble methanol	11.5 grams	Light green

From Table 03. shows that after the liquid-liquid partition is formed 2 layers are formed, namely the layer

n-hexane soluble as much as 10 grams, and which dissolves methanol by 11.5 grams. In layer chromatography

Table 04. Thin Layer Chromatography Results

No	Comparison	Separation
1.	N-heksan: Etilasetat 7:3	√

From Table 04. shows that after the TLC process is carried out with several solvent comparisons, at found that the best stain separation was found in the ratio of n-hexane: ethyl (7: 3) solvents.

Table 05. Vacuum Liquid Chromatography Results

Fraction	Eluent Comparison (n-hexane: methanol) v / v	Rf value
F1	60:40	0,74

From the table above shows that after the KCV process is obtained that the fractions F1, F3, F4, and F5, do not indicate the spot stains. And for the F2 fraction, it shows stain spots with an Rf value of 0.74

DISCUSSION

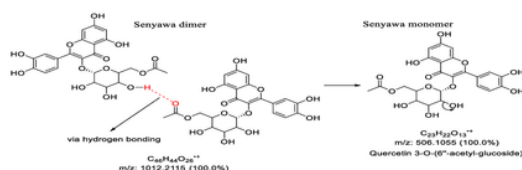
The extraction process of waru bark is done using the maceration method. Maceration is one method of separating compounds by immersion using organic solvents at room temperature. The choice of the maceration method in this study is based on the physicochemical properties of flavonoid compounds that cannot stand heating. In addition, flavonoids are easily oxidized at high temperatures (Koirewoa, 2017). Based on the results of extractions that have been done in getting a thick extract of 50 grams with a weight of 10% rendition. This is in accordance with that explained by the Ministry of Health of the Republic of Indonesia (2000) which states that the range of % *rendamen* from the extraction process that takes place perfectly is 10% -15% so that it can be said that the extraction process with maceration methods carried out in this study took place perfectly. Phytochemical screening aims to determine the types of secondary metabolites found in plants because they can react specifically with certain reagents and provide an overview of the groups of compounds contained in waru (*Hibiscus tiliaceus*) plants. From the results of observations, there was a change in the colour of the extract the skin of waru stem which has been reacted with each reagent in each tube, changes in colour from green to yellow.

The thick extract of the skin of waru (*Hibiscus tiliaceus*) stem that has been obtained, then partitioned using a separating funnel. Liquid-solid partition aims to separate polar and nonpolar compounds from the extract. The principle of this

liquid-liquid partition is to use two non-intermixed recruiters whose polarity is different, namely n-hexane and methanol, this principle is known as "like dissolve like" where polar solvents dissolve polar compounds while non-polar solvents will dissolve nonpolar compounds (Panigoro, 2017).

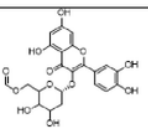
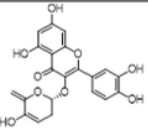
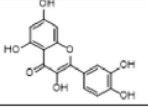
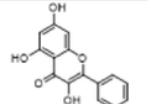
Next is the TLC stage. From the results of the previous TLC test, namely by using the mobile phase N-hexane: Ethyl acetate (70:30). Then the plates are aerated within a few minutes. Furthermore, stain spots that appear are observed using UV lights 254 nm and 366 nm. Furthermore, it was observed the smear of stain that appeared on the TLC plate. The results of the appearance of the stain showed the same stain with the Quercetin tricycle (comparative stain). Furthermore, the stain formed on the KLTP plate is scraped. The results of the scrapings are then dissolved and filtered to separate the filtrate and silica gel. After that, the resulting filtrate is then evaporated. In this study, the qualitative evidence of whether or not there are quercetin flavonoid compounds and other flavonoid derivatives thought to be contained in waru bark samples which according to some positive references contain these compounds. This identification was carried out using the Liquid Chromatography-Mass Spectroscopy (LC-MS) instrument Electrospray Ionization (ESI) system with acetonitrile and acetonitrile 10% gradient mobile phases, using a stationary phase column namely ACQUITY UPLC BEH C18 Column, with a size of 2.1 mm X 50 m and with a flow rate of 0.3 ml / minute.

Flavonoid compounds found in waru leaves are derivatives of the compound Quercetin (2- (3,4-dihydroxyphenyl) -3,5,7-trihydroxy-4H-chromen-4-one). In this MS spectrum shows the peak ion $[M + H]^+$ of 507,3483 m / z, which corresponds to the relative molecular mass of the compound Quercetin 3-O- (6'' - acetylglucoside) of 506,1055 g / mol. The compound Quercetin 3-O- (6'' - acetylglucoside) is a fractional monomer of the dimer compound Quercetin 3-O- (6'' - acetylglucoside) with hydrogen bonds

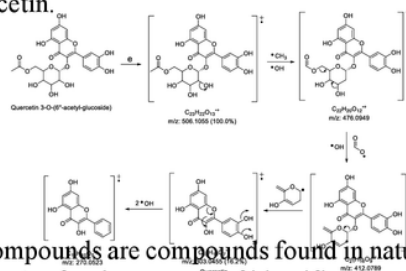


Picture of Monomers and Dimers of Quercetin Compound Derivatives
Interpretation Table of MS Spectrum Fragmentation in Waru Leaves

Peak	$[M+H]^+$	Rumus Molekul	Mr (g/mol)	Rumus Struktur	Nama IUPAC	Referensi
1	507,348 3	C ₂₃ H ₂₂ O ₁₃	506,1055		Quercetin 3-O- (6''-acetylglucoside)	(Jang et al., 2016)

2	477,322 0	C ₂₂ H ₂₀ O ₁₂	476,0949		((6R)-6-(2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl)oxy)-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methyl formate	
3	413,294 3	C ₂₁ H ₁₆ O ₉	412,0789		(R)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-((5-hydroxy-6-methylene-3,6-dihydro-2H-pyran-2-yl)oxy)-4H-chromen-4-one	
4	304,303 0	C ₁₅ H ₁₀ O ₇	303,0455		Quercetin	(Jang et al., 2016)
5	271,175 1	C ₁₅ H ₁₀ O ₅	270,0523		3,5,7-trihydroxy-2-phenyl-4H-chromen-4-one	

Based on the data in Table 1. Interpretation of the MS spectrum in waru leaves shows peak ion $[M + H]^+$ of 507,3483, this corresponds to the relative molecular mass of the compound Quercetin 3-O- (6'' - acetyl-glucoside) of 506, 1055 g / mol with the molecular formula C₂₃H₂₂O₁₃. Similarly, the peak ion $[M + H]^+$ 304,3030 m / z, which shows the relative molecular mass of the compound Quercetin that is equal to 303,0455 g / mol. The process of fragments of polymers into monomers to become structures with relatively smaller molecular masses is called fragmentation. The following is a fragmentation of the flavonoid group derivative of Quercetin.



Marker compounds are compounds found in natural materials and detected for special purposes (eg for the purpose of identification or standardization) through research (Patterson, 2006). Compounds or marker substances can also be used to mark or as identify compounds of certain plant simplicia. Selection of marker compounds is based on varieties of different factors including stability, ease of analysis, time and cost of analysis, therapeutic effects, indicators of product quality or stability or previous users by other studies.

Based on the results of the interpretation data above, it can be said that the marker compound in the sample of waru bark (*Hibiscus tiliaceus*) indicated that the Quercetin compound derivative in the MS spectrum shows a peak of $[M + H]^+$ ion of 507,3483 m / z (+4 protonation instrument positive ion mode setting), which corresponds to the relative molecular mass of the Quercetin 3-O- (6'-acetyl-glucoside) of 506,1055 g / mol. The compound Quercetin 3-O- (6''-acetyl-glucoside) is a fractional monomer of the dimer compound Quercetin 3-O- (6''-acetyl-glucoside) with hydrogen bonds with high abundance intensity. This increase in molecular weight is caused by a positive protonation mode which can potentially be that the compound can bind one or more hydrogen in the instrument so that the molecular weight increases with the number of hydrogen molecules bound by the analyte.

CONCLUSION

The results of the identification of the structure of the marker compound isolate using Liquid Chromatography-Mass Spectrometry by showing that the compound on the bark of waru (*Hibiscus tiliaceus*) is a quartetin derivative flavonoid compound namely the compound Quercetin 3-O- (6''-acetyl-glucoside) with a relative molecular mass of 507,3483

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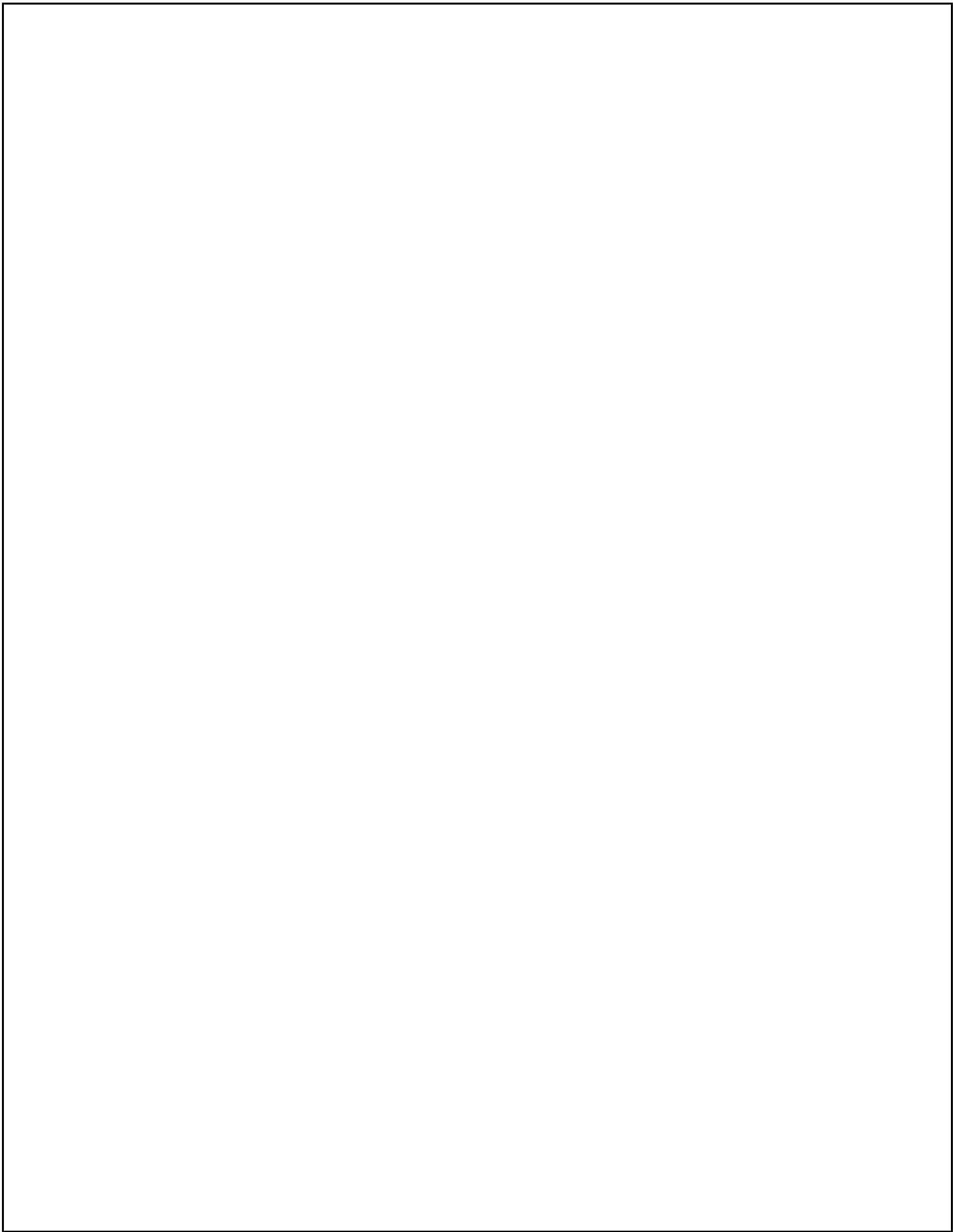
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