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EXTRACTION AND IDENTIFICATION OF ANTIMICROBIAL COMPOUNDS FROM INDIAN SPICES FOR PRESERVATION OF FRESH FRUIT CUTS

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Antimicrobial activity of essential oil extracted from some common Indian spices has been investigated against nine fruits' micro-flora. Essential oil was steam distillated by using Clevenger's apparatus from the standardized spice mix of Cumin (*Cuminum cyminum* L.), black paper (*Piper nigrum* L.), cardamom (*Elettaria cardamomum* L.), nutmeg (*Myristica fragrans*) and cinnamon (*Cinnamomum zeylanicum*). Fruit micro-flora was collected from designed fruit basket of nine fruits containing apple, guava, grapes, pear, banana, oranges, pineapple, dates and papaya. Antimicrobial activity of essential oil was tested with varying concentrations 10 µl, 15 µl, 20 µl, 25 µl and 30 µl by measuring turbidity with colorimeter at 590 nm. It was investigated from results that 30 µl concentration of essential oil was effective for inhibition of micro-flora. Chemical analysis and identification of compounds from essential oil was performed using a SHIMADZU GCMS QP-2010 gas chromatography with mass spectrometer. Total 88 compounds were identified, major were 1.4176% α-pinene, 1.3090% β-myrcene, 5.0196% β-pinene, 2.0919% sabinene, 5.6892% Benzene, 1-methyl-4-(1-methylethyl)-(CAS) p-Cymene, 21.4922% cineole, 8.2723% γ-Terpinene, 2.0483% 4-terpineol, 1.5038% α-terpineol, 12.5145% benzaldehyde, 4-(1-mthylethyl)-(CAS) Cuminic aldehyde, 2.3185% cinnamaldehyde, 2.6949% 2-caren-10-al, 2.0653% 1-phenylpropanol, 16.1015% α-terpinenyl acetate, 2.9508% 3-allyl-6-methoxyphenol, 2.1321% trans-caryophyllene. Results speak that spices can be serve as natural preservative along with as flavor, color and antioxidant additive for fresh cut fruits.

Keywords: Antimicrobial activity, Steam distillation, Essential oil, GC-MS, Fruit micro-flora

INTRODUCTION

A spice is a dried seed, fruit, root, bark or vegetative substance used in nutritionally insignificant quantities as a food additive for the purpose of flavoring. Spices are mostly used as flavoring agents in a number of foodstuffs, such as curries, bakery products, pickles, processed meats, beverages, liqueurs, etc. Naturally occurring compounds in spices such as sulpur compounds, terpenes and terpene derivatives, phenols, esters, aldehydes, alcohols and glycosides have shown antimicrobial functions (Russel *et al*., 1991). Culinary spices and herbs contain a wide variety of active phytochemicals (including flavonoids, terpenes, polyphenols, curcumins, coumarins) and may fulfill more than one function in any food to which they are added (Fabio *et al.,* 2003). Spices also contain fiber, proteins, sugars, cations and pigments (cartenoids, chlorophylls, etc.). Many spices also possess important physiological and medicinal properties. Phenolic compounds, as are vanillin, galic acid, caffeic acid, etc., are involved in olfactory, taste and tactile sensations and volatile compounds such as essential oils

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(Skerget *et al.,* 2005). The spice oils or oleoresins, which contain all the active principles of spices, are obtained by the steam distillation of ground spices (Shakuntala Mane *et al.,* 2008). It is a special type of distillation or a separation process for temperature sensitive materials like oils, resins, hydrocarbons, etc. which are insoluble in water and may decompose at their boiling point. Analysis of Essential Oil is done by using Gas Chromatography with Mass Spectrometer. The qualitative and quantitative analysis is done to know the constituents in the oil and the percentage of components present in the oil respectively, by doing so we can know the purity of that particular oil (Williams *et al.,* 1993). It is believed that Gram-positive bacteria were more sensitive to inhibition by plant essential oils than the Gram-negative bacteria.

Fresh cut fruits are healthy food and can be made easily available and ready to eat. Taste of these fruits can be enhanced by adding the flavor of spices; also preservation by inhibiting the microbial growth is possible. Trend of ready to eat food is increasing day by day but there are certain limitations to the shelf life of fresh cuts like microbial spoilage, desiccation, discoloration or browning, bleaching, textural changes and development of off-flavor or off-odor. In such cases chemical, natural or nature identical additives are preferred to maintain the characteristics of minimally processed products close to their raw unprocessed material. The benefits involved in the use of food additives are many, such as increased shelf-life, quality, nutritive value and economic saving. Though there is some risk involved in the use of chemical (synthetic) food additive. The toxic effects observed with some synthetic food additives when used at higher levels. The most commonly used preservatives are potassium sorbate and sodium benzoate. However, consumer demand for natural origin, safe and environmental friendly food preservatives is increasing. Natural antimicrobials such as bacteriocins, lactoperoxidase, herb leaves and oils, spices, chitozan and organic acids have shown feasibility for use in some food products (Corbo *et al.,* 2009). Some of them have been considered as Generally Recognized As Safe (GRAS) additives in foods. Cinnamon as an antimicrobial agent has been used in fresh cut apple slices. It was found that cinnamic aldehyde has greater antimicrobial activity than potassium sorbate (Muthuswamy *et al.,* 2008).

The main factors that determine the antimicrobial activity of spices for fresh cut fruits are the type of spice, amount used, type of microorganism, composition of food, pH value, temperature of the environment and protein, lipid, salt and sugar present in food. Furthermore the inhibitory effects of spices are mostly due to volatile oil content present in their composition. In view of this the present research includes extraction, identification of antimicrobial compounds from Indian spices in composite form and its application to enhance the shelf life of fresh fruit cuts.

MATERIALS AND METHODS

Natural and good quality spices like black pepper, cardamom, cinnamon, nutmeg, cumin and clove were procured from Kerala (India). Nine types of natural and firm fruits (apple, banana, pear, guava, orange, pineapple, grapes, papaya and dates) were purchased from local market of Nagpur. Aluminum boxes and oxywrap cling film were used to store the fresh cut fruits during study.

Size Reduction of Spices

Size reduction of spices was carried out by using electric grinder for efficient extraction of essential oil. Grinding of dried plant materials involves subjecting the whole or coarsely broken feedstock to considerable shear forces in specially designed machinery with fixed and high speed moving parts with the inevitable production of heat. Friction between the parts of the grinding head and the plant material itself usually results in temperatures of between 40 °C and 95 °C, depending on the fineness of the grinding and the nature of the starting material. Generally, finer the grind, the higher the temperature created in the mill head (and greater volatile losses), but this is offset by the more readily availability of the flavor and increased ease of incorporating the grind spice into a food mix. Prepared coarse spice mix was packed and sealed polyethylene bags and stored in dry place during study.

Isolation of Fruit Micro-Flora

Fresh fruit cuts were allowed to spoil for maximum growth at room temperature for 48 hours. Micro-flora was isolated from spoiled cut fruits. In aseptic conditions, micro-flora was scraped from the surface of fruits with the help of inoculating loop. Isolated microbial sample was used to study the antimicrobial activity of essential oil.

Standardization of Spice Mix for Fruits

Spice mixes from six Indian spices were prepared by varying the percentage content of some spices in each mix. According to the desirable flavor with combination of fruits the spices percentage was varied. Five mixes were prepared among which one is selected according to the sensory evaluation of fruits and spices for extraction essential oil.

Sensory Evaluation of Developed Mix Over the Fruits

Sensory evaluation was done by ten semi trained panel members. Parameters like taste, appearance, after taste, flavor acceptability and overall acceptability were judged by the panel members. Scoring was based upon the nine numbers hedonic scale.

Extraction of Essential Oil

The experiment was conducted in a Clevenger's Apparatus. Apparatus consist of one round bottom flask of 1000 ml in which 100 gm spice mix powder and distilled water up to half of the flask were taken. The flask was connected to a triangular arm and then condenser. Essential oil and water were separated through cork given at the end of triangular assembly. Water is allowed to boil for 5-6 hours. Oil distillation ceased was observed. Separated oil was collected and stored in cuvette below 4 °C. Steam distillation of spices is relatively an economic process to operate at a basic level and properties of oils produced by this method are not altered. This method apart from being economical, it is also relatively faster than other methods.

Gas Chromatography and Mass Spectrometric Analysis of Essential Oil

Analysis of Essential Oil was performed using a SHIMADZU GCMS QP-2010 Gas Chromatography with Mass spectrometer having mass selective detector and a HP-5 capillary column (30 m× 0.25 mm, film thickness 0.32 µm). Helium at a flow rate of 1.89 ml/min was used as carrier gas.

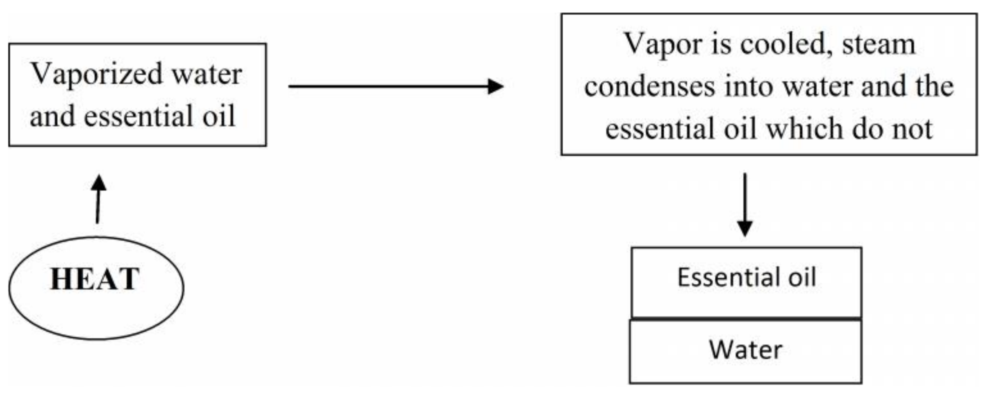
Antimicrobial Activity of Essential Oil

The in vitro antimicrobial activity was evaluated using isolated fruit micro-flora. For the further investigation, 1 ml of sample from the collected micro-flora was inoculated in test tube containing 9 ml sterile nutrient broth. This test tube was incubated at 37 °C for 24 hrs and was referred to as seeded broth. Stock was serially diluted up to 10 fold in 9 ml peptone water. 1 ml of the 10^{th} dilution sample was added into the six test tubes containing equal amount of nutrient broth (10 ml). In order to estimate the antimicrobial activity of essential oil, it was poured in the nutrient agar test tube at different concentrations (10 µl, 15 µl, 20 µl, 25 µl and 30 µl). Control sample without adding essential oil was also prepared. Test tubes were incubated at 37 °C for 24 hrs and examined for microbial growth inhibition. The turbidity was measured by observing optical density with colorimeter and results were recorded.

Determination of Cell Mass by Turbidity Measurement

Most widely used technique of measuring cell mass is by observing the light- scattering capacity of the sample. A suspension of unicellular organism is placed in a colorimeter or spectrophotometer and light is passed through it. The amount of the light absorbed is proportional to the mass of cells in the path of light. When cells are growing exponentially, increase in cell mass is directly related to cell number. This is a rapid and accurate method to estimate dry weight or cell number in unit volume, provided a standard curve is first prepared. A standard curve can be prepared by measuring bacterial growth simultaneously by two methods, and then establishing a relationship between the values obtained. Thus, by indirectly measuring the turbidity of the

Figure 1



suspension, cell weight or cell number can be determined curve (Powar *et al.*, 1992). In this experiment, microbial growth was calculated in terms of concentration (turbidity) by taking optical density using colorimeter (Photo Electronic Instruments Pvt. Ltd. Jodhpur.Cat No: 015 Sr. No. 02723) and graph was plotted. Calibration was done by distilled water. Antimicrobial activity of essential oil was determined by measuring the optical density measured at 590 nm. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of essential oil that completely suppressed microbial growth.

Statistical Analysis

During study all experiments were performed and parameters were calculated three times (unless indicated otherwise) for one sample. Mean of these three replicates was reported as result. Deviation in these readings was mentioned by calculating standard deviation for each value and represented with results.

RESULTS AND DISCUSSION

Standardization of Spice Mix for Fruits

Six commonly used spices (black pepper, cardamom, nutmeg, cumin, cinnamon and clove) are used for standardization of spice mixes. Composition of spice mix was standardized in ordered to enhance the flavor of fruits, as well as acceptability and shelf life of fresh cut fruits. The formulation was standardized by varying quantity of spices among six selected. Spice mix was made with equal proportion of all six spices. In mix 2 and 3 slight change was made in proportions. Mix 4 and 5 were formulated by eliminating the clove. The development of mixes was carried out by evaluating the sensory properties of fruits and spices. The formulations of all five spices are shown in Table 1. In mix 1 proportion of all spices was kept constant so that each will give its flavour and taste. In mix 2 proportion of black pepper, nutmeg and clove was equal where cardamom, cumin and cinnamon were added in same proportion. Mix 3 was having alternative proportion with respect to mix 2. In mix 4 quantity of cardamom was increased up to 33% and clove was eliminated in both mix 4 and 5. Black pepper and cumin was added in equal proportion as well as nutmeg and cinnamon was added in equal quantity. Composition of cardamom and cumin was equal in mix 5 also nutmeg and cinnamon was in same quantity. Black pepper added was 18.18% in mix 5.

Table 1: Standardization and Development of Spice Mix (in Percent)

S No.	Spice Powder %	Mix 1	Mix 2	Mix 3	Mix 4	Mix 5
1	Black pepper	16.7	11.11	22.22	22.22	18.18
2	Cardamom	16.7	22.22	11.11	33.3	36.36
3	Nutmeg	16.7	11.11	22.22	11.11	4.54
4	Cumin	16.7	22.22	11.11	22.22	36.36
5	Cinnamon	16.7	22.22	11.11	11.11	4.54
6	Clove	16.7	11.11	22.22	-	-

Sensory Evaluation Sheet

Sensory evaluation of spice mix was performed and formulation was standardized to determine the most acceptable composition of mix. The mix was evaluated for taste, appearance, after taste, flavor and acceptability on nine points hedonic scale by the panel of ten semi trained members, which includes 5 male judges and 5 female judges. For sensory evaluation, 1% aqueous solution was prepared in distilled water to avoid interference of foreign matter contributing in flavor alteration. Similarly 1% mix (day) was sprinkled over the fruit. Uniformity and homogeneity was attended by mixing.

From the sensory evaluation sheet it was observed that, spice mix 5 was more acceptable in all parameters comparing others. Sample 4 as slightly liked by panel members in taste, appearance, acceptability and slightly disliked in after taste and flavor. Panel members suggested it was bitter in taste and the original taste of fruit was replaced by the strong taste of spices. Clove was dominating factor in the first three samples. To overcome this problem clove was eliminated. Replacement of clove was done by increasing the proportion of cumin and cardamom. Cumin, black pepper, nutmeg, cinnamon and cardamom were found pretty well with fruits. Among these five formulations fifth was mostly accepted by panelists. So it was finally used for extraction of essential oil. Also from the remarks it was found that, flavor of spices was giving good taste to the fruits and the combination of fruits and spices is acceptable.

Yield of Essential Oil

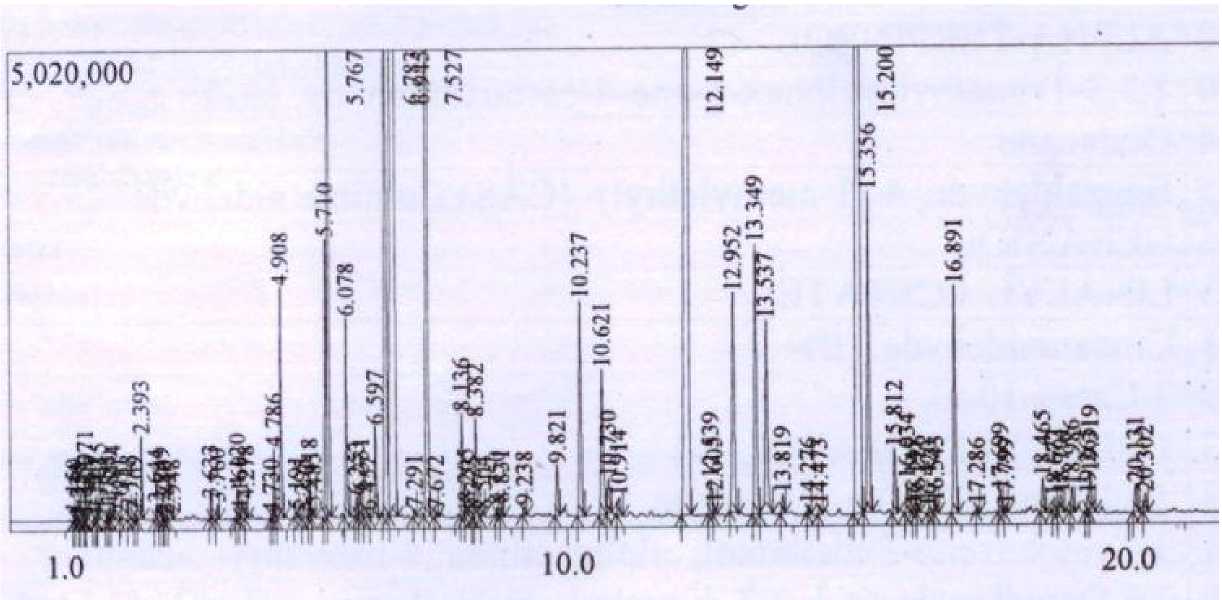
Total practical yield of essential oil was found 1.5%. From 100 gm of spices 1.5 ml essential oil was obtained. Total theoretical yield was calculated and it was 1.74%. The difference in the practical yield and theoretical was may be due to high temperature or vapor leakage. Density of

Table 2: Sensory Evaluation Data of Fruits and Spices

Parameters	S1		S2		S3		S4		S5	
	SM	SM+F	SM	SM+F	SM	SM+F	SM	SM+F	SM	SM+F
Taste	4.8±0.73	5.7±1.39	5.4±0.81	5.8±0.84	4.5±1.67	5.4±1.67	6.3±0.81	6.6±0.80	6.3±1.59	6.71±1.48
Appearance	5.4±1.01	4.9±1.57	5.7±0.98	5.9±0.58	5.5±1.16	5.5±0.88	6±0.81	5.8±1.28	7.2±0.74	7±1.67
After taste	5.5±1.22	5.1±1.59	5.7±1.12	5.6±1.13	4.8±1.96	4.6±1.76	6±1.11	6.1±1.28	6.5±1.5	6.8±0.74
Flavor	5.2±1.22	5.2±1.29	5.5±0.92	5.4±1.24	4.6±1.54	4.7±1.53	5.5±1.64	4.8±1.89	6.1±1.46	6.5±1.38
Acceptability	5.6±1.23	5.0±1.64	5.6±0.6	6.8±0.87	4.8±1.34	5.1±1.82	5.8±1.46	6.2±1.69	6±1.41	7±1.15
Overall acceptability	5.6±1.06	5.4±1.35	5.8±1.09	5.5±1.25	5.1±2.41	5.9±1.87	6.5±1.07	6.6±1.56	6.8±1.21	7.3±0.74

Note: SM - spices mix and SM+F - spices mix on fresh cut fruits; Mean values ± SD are presented, n = 10.

Figure 2: Chromatogram of Essential Oil



extracted essential oil is 0.9909 gm/ml it was calculated by mass and volume ratio.

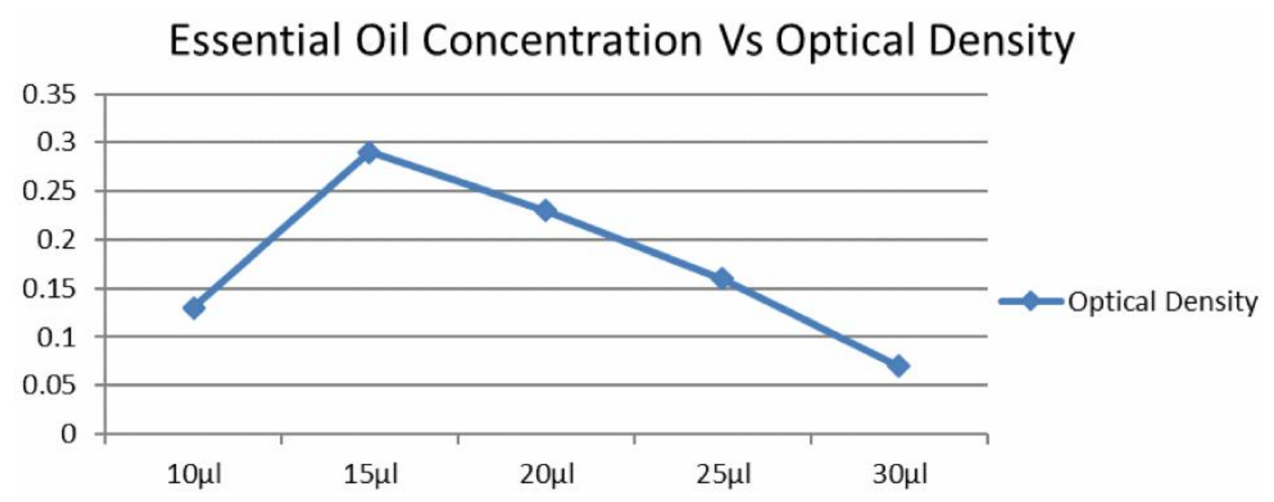
GC-MS Analysis

The standardized spice mix for extraction of essential oil included black pepper, cardamom, nutmeg, cumin, and cinnamon. In which amount of cardamom and cumin was higher, i.e., 36.36%, nutmeg and cinnamon was 4.54%, it was low compared to others and 18.18% of black pepper. Clove was not included as it was giving bitter taste and dull appearance to the fruits, this condition was not accepted by panel members. The steam distillation of this formulated spice mix resulted in the essential oil containing essential components of these all spices individually. For the identification of these components GC-MS analysis was performed. Total eighty eight compounds were characterized with this oil. The analysis shows the composition of the oil and also the percentage of each component. Graph generated by Gas chromatography-Mass Spectrometer apparatus shows the peaks and retention time of each

Table 3: Peak Report-Constituents of Essential Oil

Peak	R. Time (min)	Area	Area %	Name
30	4.908	4327049	1.4176	Alpha.-pinene
35	5.71	638527	2.0919	Sabinene
36	5.767	15321809	5.0196	Beta-pinene
37	6.078	3995628	1.309	Beta-myrcene
42	6.783	17365448	5.6892	Benzene, 1-methyl-4-(1methylethyl)-(CAS) p-cymene
43	6.945	65602252	214922	Cineole
45	7.527	25250140	8.2723	Gamma- terpinene
56	10.237	6252257	2.0483	4-terpineol
57	10.621	4590243	1.5038	Alpha-terpineol
60	12.149	38192936	12.5145	Benzaldehyde, 4-(1-methylethyl)-(CAS) cuminicaldehyde
63	12.952	7076823	2.3185	Cinnamaldehyde
64	13.349	8225972	2.6949	2-Caren-10-al
65	13.537	6304003	2.0653	1-phenylpropanol
69	15.2	49147639	16.1015	Alpha- terpinenyl acetate
70	15.356	9006938	2.9508	3-allyl-6-methoxyphenol
77	16.891	6507870	2.1321	Trans-caryophyllene

Figure 3: Antimicrobial Activity of Essential Oil at Different Concentrations



component. It was indicated in Figure 2. List of compounds identified after analysis containing peak number, retention time, area covered by peak, percent area and name of identified compound are reported in Table 3.

The results obtained from the GC-MS report included the retention time, area, area % and names of compound detected. From the total eighty eight compounds major compounds were 1.4176% α-pinene, 1.3090% β-myrcene, 5.0196% β-pinene, 2.0919% sabinene, 5.6892% Benzene, 1-methyl-4-(1-methylethyl)-(CAS) p-Cymene, 21.4922% cineole, 8.2723% ã-Terpinene, 2.0483% 4-terpineol, 1.5038% α-terpineol, 12.5145% benzaldehyde, 4-(1-mthylethyl)-(CAS) Cuminic aldehyde, 2.3185% cinnamaldehyde, 2.6949% 2-caren-10-al, 2.0653% 1-phenylpropanol, 16.1015% α-terpinenyl acetate, 2.9508% 3-allyl-6-methoxyphenol, 2.1321% trans-caryophyllene. The results were found identical with Manuel Viuda-Martos *et al.*, he observed the principal components of Cumin essential oil was manly composed of γ-pinene (27.4%), p-cymene (20.49%) and cuminal (20.39%). Also as reviewed by Lopez-Mato *et al.*, cinnamic aldehyde is antimicrobial compound that have been identified from cinnamon. As stated above Naturally occurring compounds in spices such as sulpur compounds, terpenes and terpene derivatives, phenols, esters, aldehydes, alcohols and glycosides have shown antimicrobial functions (Russel *et al*., 1991). Similar compounds were obtained from this study and the compounds identified come under these categories.

Antimicrobial Activity of Essential Oil

In this study essential oil was extracted from spices to use it as antimicrobial agent against pathogens responsible for spoilage of fruits. The antimicrobial effect of spices as already studied and results are reported in literature. Before using essential oil as antimicrobial agent it is necessary to study the antimicrobial effect of this essential oil. The activity of oil was tested against fruit micro-flora.

When microbial sample with different concentrations of essential oil was incubated, after incubation it was observed that turbidity of all samples was different. To know the effectiveness of antimicrobial essential oil cell mass concentration was analyzed by turbidity measurement. The optical density was measured for each sample at 590 nm. Results obtained are mentioned in the Table 4 and graphical representation of optical density Vs essential oil concentration is shown in Figure 3.

Table 4: Colorimeter Analysis to Study the Antimicrobial Effect of Essential Oil

Concentration of Essential Oil	Optical Density
10 µl	0.13
15 µl	0.29
20 µl	0.23
25 µl	0.16
30 µl	0.07

Light is passed through the suspension of microorganism and essential oil then all light that is not absorbed is radiated. Light passing through a suspension is absorbed, and the amount of absorbed is an indication of the biomass present in the suspension. Less light absorbed will give lower optical density/ absorbance and finally low amount of microbial concentration. The results say that at low concentration of essential oil (10 µl and 15 µl) microbial growth was observed. It means that this concentration of essential oil is not enough to stop the growth of fruit micro flora. As the concentration increases the value of optical density decreases i.e. turbidity decreases. Turbidity is the indication of microbial growth. At higher concentration of essential oil the microbial activity gets stopped. From this experiment 30 µl concentration of essential oil was found good to show better antimicrobial activity. This means that 30 µl is the minimum inhibitory concentration to kill the fruit micro flora.

CONCLUSION

The major components in essential oil included, 1.4176% α-pinene, 1.3090% β-myrcene, 5.0196% β-pinene, 2.0919% sabinene, 5.6892% Benzene, 1-methyl-4-(1-methylethyl)-(CAS) p-Cymene, 21.4922% cineole, 8.2723% γ-Terpinene, 2.0483% 4-terpineol, 1.5038% α-terpineol, 12.5145% benzaldehyde, 4-(1-mthylethyl)-(CAS) Cuminic aldehyde, 2.3185% cinnamaldehyde, 2.6949% 2-caren-10-al, 2.0653% 1-phenylpropanol, 16.1015% α-terpinenyl acetate, 2.9508% 3-allyl-6-methoxyphenol, 2.1321% trans-caryophyllene. These are mainly responsible for the flavor, aroma and posses antimicrobial activity against various food borne pathogens. Antioxidant activity of this essential oil influences on retarding enzymatic browning of fresh fruit cuts. The ingredients obtained from this study indicate that essential oil can be fully utilized for the preservation and/or extension of shelf life of fresh cut fruits at minimum or lower concentration. Essential oil also enhances the organoleptic acceptability of the fresh cut fruits.

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